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DINOFLAGELLATE TOXINS RESPONSIBLE FOR CIGUATERA FOOD POISONING

Annual Report

Donald M. Miller

December, 20 1989

Supported by

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
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Department of Physiology, School of Medicine  
Southern Illinois University, Carbondale, Illinois 62901-6521

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## SUMMARY

Ciguatera is a syndrome occurring in humans who have become intoxicated from eating poison fish. Fish spontaneously accumulate the toxin through the food chain or directly from eating toxic dinoflagellates. Previous research points to the presence of multiple toxin involvement. Some of these toxins are porported to be ion channel inhibitors or activators. In addition to the establishment of facilities, this contract requires the growth of sufficient quantities of three different species of dinoflagellates to allow purification of milligram quantities of toxins for delivery to the U.S. Army Medical Research and Development Command. In this third year of the contract, growth of the dinoflagellate *Gambierdiscus toxicus* in mass culture has been successful beyond previous projections and expectations. Purification of the products of one of these toxins down to the 1 µg/MU level in milligrams quantities has been achieved. Further purification and NMR analysis is still in progress.

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## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

## TABLE OF CONTENTS

Front Cover .....	i
Reverse of Front Cover .....	ii
Report Documentation Form, DD Form 1473 .....	iii
Summary .....	1
Foreword .....	2
Table of Contents .....	3
Table of Figures .....	4
Table of Tables .....	5
Body of the Report .....	6
Statement of the Problem .....	6
Background .....	7
Ciguatera Poisoning .....	7
The Ciguatera Syndrome .....	7
Multi-toxin Involvement .....	7
Dinoflagellates Producing Toxins .....	8
Dinoflagellate Toxins Affecting Ion Channels .....	8
Dinoflagellate Involvement in Ciguatera .....	8
Strains of Toxin Producing Dinoflagellates .....	9
Isolation of Dinoflagellate Toxins .....	9
Fish Toxins .....	11
Significance .....	13
Approach to the Third Year .....	14
Methods for Growth of Cells .....	14
Chemosystematic Studies .....	15
Toxin Potencies .....	18
Mass Culture of Dinoflagellates .....	20
Mouse Bioassay .....	20
Ileum Assay Procedures .....	21
Tests for Purity of Toxins .....	24
NMR Spectroscopy .....	25
Packaging of Toxins for Shipment .....	25
Acquisition of Toxic Dinoflagellates .....	25
Overall Objectives for the Third Year .....	25
Results .....	26
Maximizing Toxin Production .....	35
Mass Culture of Dinoflagellates .....	35
Purification of Extracts .....	37
Separation of GT-175 Material .....	37
Separation of GT-350 Material .....	46
Brevetoxin Antibody Competition .....	50
NMR Spectroscopy .....	52
Deliveries .....	54
Acquisition of Toxic Dinoflagellates .....	55
Discussion .....	55
Literature Cited .....	60
Glossary .....	68
Distribution List .....	69
Back Cover .....	70

## TABLE OF FIGURES

Figure 1.	Extraction Procedures .....	10
Figure 2.	Guinea Pig Ileum Setup .....	21
Figure 3.	Reversible Assay Protocol .....	23
Figure 4.	Non-reversible Assay Protocol .....	24
Figure 5.	Reversible and Non-reversible Assay Protocol .....	24
Figure 6.	Reproduction Rate vs Light Intensity .....	27
Figure 7.	Protein vs Light Intensity .....	28
Figure 8.	Lipid vs Light Intensity .....	29
Figure 9.	Carbohydrate vs Light Intensity .....	29
Figure 10.	Chlorophyll a vs Light Intensity .....	30
Figure 11.	Chlorophyll c vs Light Intensity .....	30
Figure 12.	Peridinin vs Light Intensity .....	31
Figure 13.	Diadinoxanthin vs Light Intensity .....	31
Figure 14.	Dinoxanthin vs Light Intensity .....	32
Figure 15.	Potency vs Light Intensity .....	33
Figure 16.	Cell vs Potency .....	33
Figure 17.	Ammonium vs Potency .....	34
Figure 18.	Mouse Units Produced during Third Year .....	36
Figure 19.	Mouse Units for Entire Contract .....	37
Figure 20.	Liquid-liquid Partitioning GT-175 .....	38
Figure 21.	ACN Precipitation of GT-175 .....	39
Figure 22.	Brevetoxin Competition Experiment .....	51
Figure 23.	NMR Spectra of MTX-2 .....	53

**TABLE OF TABLES**

Table 1.	Physiological Saline Components .....	22
Table 2.	Correlation Matrix Date of Growth Experiments .....	34
Table 3.	Harvests of Dinoflagellates .....	35
Table 4.	Growth Production Data .....	35
Table 5.	Ileum Assays of MTX .....	39
Table 6.	Low Pressure Chromatography of ESMF .....	40
Table 7.	Summary of Purification Factionation .....	43
Table 8.	Products of Extraction and Purification .....	45
Table 9.	Hexane Methanol Separation .....	47
Table 10.	ACN Extraction .....	47
Table 11.	Ethanol Precipitation .....	48
Table 12.	Preparative Separation .....	48
Table 13.	Hexane Methanol Separation .....	49



## BODY OF THE REPORT

### STATEMENT OF THE PROBLEM

Ciguatera is one of several forms of food poisoning which occurs in humans resulting from the ingestion of toxic fish. The "ciguatera syndrome" is the result of toxins accumulated by fish through the food chain from dinoflagellates. Dinoflagellates produce a variety of toxins, some of which are ion channel inhibitors. In the case of ciguatera, the toxins can be accumulated through the food chain and stored by fish which are eventually consumed by humans. The ingestion of toxic fish produces a variety of digestive and neurological symptoms and sometimes death. At the present time, there is no adequate assay system for the detection and identification of the toxins. The structure and mode of action of these toxins is unknown. However, published results on crude toxins have described them as sodium and calcium channel inhibitors.<sup>[1,2]</sup> There is at present no known prophylactic or ameliorating treatment for ciguatera intoxication, notwithstanding a recent popular report of manitol therapy.<sup>[3]</sup>

The acquisition of milligram amounts of purified toxins would help to unravel the molecular structure of the toxins, their physiological actions and help to develop effective prophylactic treatment and effective countermeasures against the actions of the toxins. Our previous work on "ciguatera" (funded by the U.S. Food and Drug Administration and College Sea Grant Program) resulted in the establishment of the SIU culture collection of toxic dinoflagellates. We proposed to grow three species of toxic dinoflagellates in mass culture for an extended period of time. After the cultures reached maximum growth they would be harvested and crude extracts made. The crude extracts would be assayed by mouse (LD<sub>50</sub>) and an isolated bioassay preparation. Crude extracts would be purified by HPLC. The purified toxins would be assayed the same as the crude and samples of purified toxin delivered to the US Army Medical Research Institute of Infectious Diseases.

## BACKGROUND INFORMATION

### Ciguatera poisoning

Ciguatera poisoning is a syndrome which occurs following the ingestion of certain tropical marine reef-fishes that sporadically acquire toxicity. It is one of nine known forms of ichthyosarcotoxism: poisoning of humans resulting from eating fishes which contain poison within their musculature, viscera or skin.<sup>[4]</sup> Halstead<sup>[5]</sup> has implicated over 400 species of marine fishes as carriers, most, if not all, of which are an integral part of the food web of coral reefs associated with oceanic islands within a circum-global belt from 35°N to 34°S.<sup>[5,6]</sup>

### The ciguatera syndrome

The symptoms that occur after eating toxic fish typically include both gastrointestinal and neurological manifestations. Typical symptomatology in humans has been reviewed and summarized by several authors.<sup>[7-9]</sup> Earliest symptoms of intoxication usually include gastro-intestinal upset, which may last for several hours or weeks. Moderate to severe intoxications usually produce neurological symptoms which may last weeks to months. Irregularities in nerve conduction parameters in fish,<sup>[10,11]</sup> mammals<sup>[12]</sup> and humans<sup>[13,14]</sup> have been documented. Thus, it is quite clear that intoxication affects the nervous system for extended periods of time. In at least one case of severe intoxication symptoms persisted for 25 years:<sup>[15]</sup> death may result and if so, it usually occurs within several days. In an isolated case, death has occurred within ten minutes and the fatality rate has been approximated as 12%<sup>[5]</sup> and 3%.<sup>[8]</sup> It is suspected that a large number of ciguatera intoxications, some from eating frozen fish, are not recognized as such.<sup>[16]</sup>

### Multi-toxin involvement

A few early researchers suggested that the great variety of symptoms displayed by patients suffering from ciguatera and their

inconsistent responses to certain clinical treatments indicated that there was more than one primary toxin causing the ciguatera syndrome,<sup>[17,18]</sup> while Banner et al.,<sup>[19]</sup> argued that ciguatoxin was the principal factor. Nevertheless, later studies coupled with the variability in results from testing of extracted fish tissues on a variety of preparations have emphasized the occurrence and importance of multiple toxins.<sup>[20-25]</sup> Yasumoto et al.,<sup>[22]</sup> provided evidence that ciguatoxin was of exogenous origin and was not a metabolic product of primary consumers. These authors reported that an analysis of gut contents of *Ctenochaetus striatus* (a detrital feeder, exclusively) revealed a portion, designated as unidentified particles, containing a high concentration of "ciguatoxin". A recent report has indicated that ciguatoxin from both fish and dinoflagellate cells are modified forms of brevetoxin.<sup>[26]</sup> A second recent report has indicated that the toxin detected in ciguatoxic fish (mackerel) was palytoxin.<sup>[27]</sup> Nevertheless, no complete structure for either ciguatoxin or maitotoxin has been published.

#### Dinoflagellate toxins affecting ion channels

The use of ion channel toxins has been crucial to uncovering the mechanisms of how ion channels work. It is well documented that two other toxins from dinoflagellates, saxitoxin and gonyautoxins are inhibitors of sodium channels. Brevetoxins have been found to affect sodium ion channels.<sup>[1,28]</sup> The current literature is somewhat confusing and indicates that ciguatera toxins act on sodium channels or on calcium channels or both.<sup>[1,2]</sup>

#### Dinoflagellate involvement in ciguatera

That dinoflagellates are the source of ciguatera-toxins has been well documented. In the Pacific, Yasumoto and others<sup>[29,30]</sup> obtained significant quantities of ciguatoxin from samples of detritus collected from dead coral near the Gambier Islands. The most toxic fraction of the detritus contained large numbers of a dinoflagellate, which he tentatively identified as "*Diplopsalis* sp." Subsequently, Adachi and Fukuyo<sup>[31]</sup> named the organism *Gambierdiscus toxicus*. Yasumoto et al.,<sup>[21]</sup> connected the production of toxin with both the

dinoflagellate and toxic effects in mice.<sup>[32]</sup> The dinoflagellate, *G. toxicus* has subsequently been isolated from ciguatera prevalent areas near Japan,<sup>[32,33]</sup> and Hawaii.<sup>[34,35]</sup> McFarren and others<sup>[35]</sup> have provided accounts of ciguatera-like poisoning (*G. breve* ?) from shellfish collected from the west coast of Florida. Other investigators have published on *Gambierdiscus toxicus* from Florida.<sup>[36]</sup> Tindall and his group<sup>[37]</sup> have grown in mass culture and extracted toxins from three dinoflagellate species which were isolated from areas of the Caribbean in which ciguatera intoxication was prevalent. Thus far, three particular dinoflagellates are implicated in the production of ciguatera toxins: *G. toxicus* in which four toxic fractions have been identified, GT-1, GT-2, GT-3, GT-4; *P. concavum* in which five toxic fractions have been described, PC-1, PC-2, PC-3, PC-4, PC-5; and *P. rathymum*(= *P. mexicanum*) in which one toxic fraction, PR-1, has been described.

#### Toxin producing dinoflagellate strains

There is ample evidence to indicate that different strains of the same species of dinoflagellates produce different numbers and amounts of toxin.<sup>[38-40]</sup> There are also reports of loss of toxicity of dinoflagellates after culture. Under our conditions we have found that the initiation of mass cultures from unialgal or pure cultures has confirmed the strain differences but our cultures (*G. toxicus*) have produced toxins through continual subculturing for a period of 10 years and through over 30+ large scale cultures. The same holds true for the other species.

#### Isolation of dinoflagellate toxins

Hashimoto<sup>[21]</sup> extracted toxic components from *G. toxicus* cells utilizing a boiling methanol extraction prior to doing a water-ether partitioning. The ether extracted portion was further treated with acetone to derive a toxic fraction. Since we have found this fraction to precipitate in very cold acetone we term it the ether soluble acetone precipitate (ESAP) fraction. Most cell isolation procedures used an initial partitioning of the cells with a water-ether mixture.

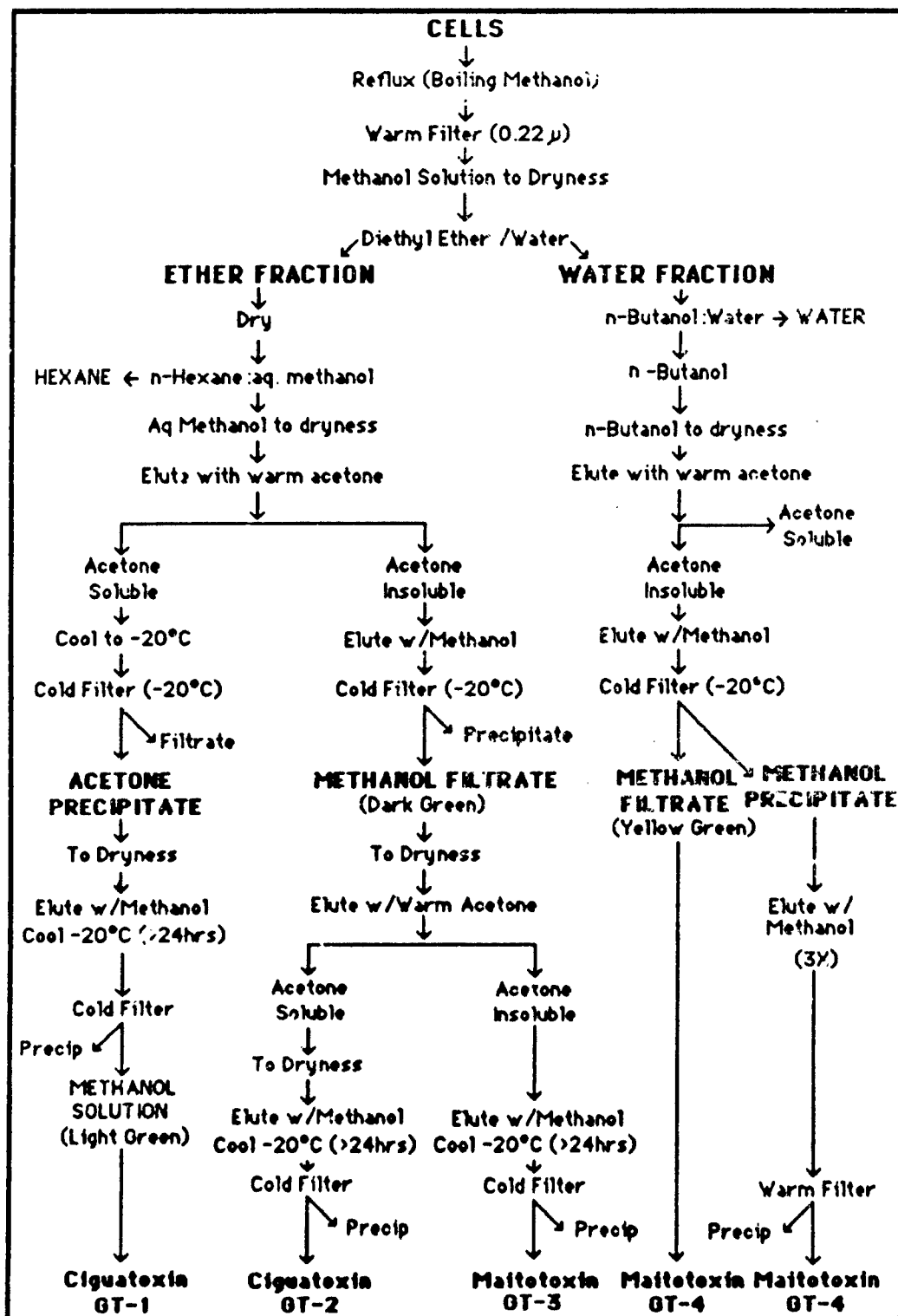


Figure 1. Flow diagram of separation procedures for *Gambierdiscus toxicus*.

The treatment of the water phase of the cell extracts have been similar by all investigators. After an acetone partitioning the filtrate is chromatographed to yield a water soluble toxic component (Figure 1).

The treatments for the ether phase of the cell extracts have differed. Yasumoto used a technique which involved an acetone extraction of the ether-water phase. Bagnis and others<sup>[41]</sup> modified the technique to include a cold acetone treatment that resulted in both ether-soluble acetone precipitates (ESAP) and ether-soluble acetone filtrates (ESAF). Even though he utilized this separation procedure, he then combined the ESAP with the WSAP fraction. Tindall and his group discovered that when the ESAF material was kept in the cold acetone all of the toxic activity eventually precipitated out of the filtrate. Thus, they adopted acetone precipitation as a step in the procedure hoping to further purify the toxic component. The toxic fractions obtained by these procedures differ with the particular dinoflagellate species.

### Fish toxins

The studies of toxins from fish extracts are difficult to draw conclusions from for several reasons, the most critical of which is that, if there are multiple toxins in fish, determined by their diet, and any one toxin may have different effects on a variety of assay systems, then it is possible that the extraction of toxin from the same species of fish by investigators from different locales will produce different symptoms.

From the foregoing we conclude, that if we want to determine if a particular fish is toxic, we must have a test(s) or assay system(s) which is specific at a known level for each of the particular toxins which may be involved in the ciguatera syndrome. For this express reason we decided at the outset of our experimentation that the utilization of cell cultures would be the most productive approach.

Indeed, the same philosophy argued above for the diagnosis of toxic fish would apply to the treatment of the disease ciguatera. A different treatment would be called for were a person intoxicated with a sodium channel inhibitor rather than a calcium channel activator. Eventually, it would be ideal to have an assay system which would, in fact, reflect the number, kinds and amounts of toxins. The construction of these specific chemical tests is only possible, however, if one has reasonably pure toxin, which is separated from other toxins and identified.

These constraints have dictated our approach to the entire problem since we started our research in 1978, as follows:

1. Identify sources of the toxins.
2. Produce large amounts of toxic organisms.
3. Improve extraction techniques.
4. Find a sensitive bioassay(s) for screening.
5. Use bioassay to assist in purification.
6. Improve purification techniques.
7. Use purified toxins to:
  - a. investigate physiology.
  - b. investigate structure.
  - c. elaborate chemical assay system.

Our preliminary works<sup>[37,42-44]</sup> have established that at a minimum at least six different toxins and possibly a seventh are involved in the ciguatera syndrome:

1. Ciguatoxin
2. Maitotoxin
3. Slow acting toxin (unidentified)
4. Scaritoxin-like toxin
5. Okadiac acid
6. Fast-acting toxin I (unidentified)
7. Fast-acting toxin II (unidentified)

Clearly, the understanding of the entire problem of the toxins produced by dinoflagellates requires a definitive test or set of tests which will allow us to discriminate between the toxins and be able to

quantify and follow them. Other desirable features of a test procedure would be 1) provide the same baseline for each of the toxins, 2) consume only a small amount of toxin, 3) require a small amount of time for the assay, and 4) be able to detect modifiers of sodium channels in membrane as well as calcium channels.

### Significance

"Ciguatera-toxins" are involved in a variety of short term symptoms for which people commonly present themselves to a doctor for treatment (e.g. diarrhea, headache, etc). In addition intoxication can result in prolonged disability or even death. Another aspect of the toxins and one which has yet to be addressed is the long term effects on animals that is: are they cumulative, are there storage sites, what are the affective sites, and how long before excretion? The reoccurrence of neurological symptoms years after intoxication would seem to indicate a retention of the toxin and/or toxic effects in the nervous system.

The toxins also include a novel calcium channel inhibitor<sup>[2]</sup> and a sodium channel inhibitor.<sup>[1]</sup> In addition, there is the prospect of discovering other new and important ion channel inhibitors. Ion channel inhibitors have been essential to our present understanding of ion channel physiology and structure. Clearly, the identification, isolation and purification of individual toxins involving ion channels will expedite (1) an understanding of their structure, (2) allow the investigation of their physiological actions, (3) expedite the formulation of ameliorative and prophylactic treatments, and (4) allow the elaboration of a specific chemical assay.



## APPROACH TO THE THIRD YEAR OF THE STUDY

### Methods for growth of cells

The dinoflagellate cultures which we used for this project are part of the Southern Illinois University Culture Collection, housed in the Department of Botany. At present this collection houses strains representing dinoflagellate species isolated from "ciguatera community" areas of the British and U. S. Virgin Islands and other recently acquired species. Our stock cultures are routinely grown in 50 ml volumes in 125 ml Erlenmeyer flasks. The medium is ES Medium<sup>[45]</sup> made with natural seawater, with 1.5% soil extract added. These cultures are kept in refrigerator-type culture chambers at 27°C and 500 ft-c. cool white fluorescence illumination (either continuous or on a 16:8 light-dark cycle, depending upon the requirements of the particular species). Stock cultures are transferred every 7-10 days. Two generations of cultures are retained as back-ups to the new transfers. The subculturing and maintenance of triplicate cultures is labor intensive and requires approximately 20 manhours per week by an experienced person. In addition, all cultures are examined periodically by one of us to check for contamination. Currently we maintain stock cultures of over 153 strains of dinoflagellates. Preparation of the growth medium requires millipore filtration (0.45 or 0.22  $\mu$ m) and sterilization of the sea water, sterilization of the flasks, compounding of the growth medium, inoculation and siting in the growth chambers. Conservatively, this requires approximately 20 manhours per week for a total of 18 carboys. Because the growth cycle for both the subcultures and the mass cultures takes four weeks, we initiate a mass culture every two weeks. It is critical for the cultures, to achieve the maximum toxin production, that they be harvested very close to the 30 day period.

The development of mass cultures involves transferring cells from stock cultures to a series of two liter fernbach flasks containing

enriched seawater medium. After the early stationary phase of growth has been reached (approximately 15-20 days) each of these cultures are used to inoculate 18 liters of the same medium in 20 liter carboys. Mass cultures are grown under the same light and temperature regime as noted above and are aerated continuously in order to prevent CO<sub>2</sub> depletion and to provide moderate agitation. Cells from small cultures are harvested by centrifugation or filtration. Cells from mass cultures are harvested by means of a Pelicon concentrator using 0.45  $\mu$ m membranes after cultures reach the early stationary phase of growth (30-35 days). If the culture has excessive amounts of slime it is first sieved before the use of the Pelicon.

#### Chemosystematic studies

Systematics is a branch of taxonomy which deals with assessing variation in characters between and within genera from living material.<sup>[46]</sup> A systematic study has been incorporated into this project as a means of identifying clones (cultures initiated from individual cells) which are inherently good producers of toxins. Our culture collection now includes more than thirty-eight clones of *Gambierdiscus toxicus* from a variety of locations including Bermuda, the Bahamas, Florida, the Caribbean, Hawaii and other areas. It is this diverse, living material which forms the basis for the systematic studies.

Our first approach in assessing clonal differences was to acclimate the *G. toxicus* clones to the same conditions (light, temperature and medium) in one liter cultures. Once acclimated, the final one liter culture were harvested and also used to inoculate an additional one liter culture which served as the starter for a 15 liter culture. The toxicities of the crude methanol cell extract from the acclimated one liter cultures were compared in terms of the number of mouse units per milligram of dried cells. This is a quantitative measure of clonal toxicity, and does not consider qualitative differences in toxins. We assert that because of the acclimation

process the varying potency of these extracts among clones is due to interclonal genetic differences and not to environmental differences.

Beginning in 1989 the extract potencies of several clones from the first and the last one liter culture were compared in order to assess the significance of the acclimation process. In addition, the potency of extracts from clone 135 were compared monthly in order to determine when acclimation was achieved and once achieved, how stable a character it is. An additional control experiment was run with the Martinique (Caribbean) clone (175).

Clone 175 has been sub-cloned so that we can assess any toxicity differences which may result from micro-environmental differences in the culture chambers. Only toxicities which exceed the methodological errors were used for "chemosystematic" comparisons ("chemo" referring to the toxins and "systematic" to the clonal comparisons). The material from the 15 liter cultures was used to assess qualitative differences in toxins among the clones.

The genetic comparisons indicated that clone 175 produces more toxin per unit weight than approximately twenty other clones of *Gambierdiscus toxicus* surveyed. Consequently, this clone was selected for physiological experiments designed to enhance toxin production, hopefully including the lipid-soluble toxin. The first phase of this work involves examining the macromolecular components and toxin production of clone 175 when grown under seven different light intensities and at three temperatures. These are 378, 648, 1081, 1999, 3350, 3782 and 4300 lux light intensities and 28°C, 25°C and 22°C temperatures.

The cultures were acclimated to each temperature following the methods of Brand et al.<sup>[47]</sup> and Bomber et al.<sup>[48]</sup> All cultures are grown in a Percival culture chamber equipped with microprocessor controls. The microprocessor enables us to change temperatures slowly using a constant gradient. The cultures were harvested through a 32  $\mu$ m screen and lyophilized for approximately 24 hours until dry.

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Kochert<sup>[49]</sup> determined protein from cell pellets that were first extracted with chloroform/methanol. Total lipids were then determined from the organic solvent extracts. We determined that as much as 10% of the protein will enter the organic solvents. Thus, we analyzed proteins and lipids separately. The dried cells were extracted with 1N NaOH with sonication followed by immediately adjusting the pH to approximately 7.5 with distilled, deionized, charcoal filtered water. This method yielded far better protein extraction than SDS (sodium dodecyl sulphate) or the method of boiling the cells in 1N NaOH<sup>[49]</sup>. The extract was filtered through a 0.2  $\mu$ m screen and analyzed for proteins by the Bio-Rad micro-method. Carbohydrates contents were determined from the same extracts via the sulphuric acid method.<sup>[49,50]</sup> Lipids were determined from separate samples following Freeman et al.<sup>[51]</sup> and Sperry and Brand.<sup>[52]</sup>

The chlorophyll content was determined from the equations of Jeffrey et al.<sup>[53]</sup> Carotenoids were determined via the methods of Jensen.<sup>[54]</sup> All samples were processed in darkened fume hoods and stored under nitrogen for best preservation. Most samples were analyzed within two weeks of harvest. The potencies were determined as before (previous quarterlies) using the linear interpolation tables of Weil.<sup>[55]</sup> Ammonium and phosphate uptake rates were determined from disappearance of the nutrients from the culture medium following the methods of Strickland and Parsons.<sup>[56]</sup>

Previous work<sup>[57]</sup> has determined that it is not possible to completely identify a systematic "variant clone" or "race" of *G. toxicus* by analyzing only one character, e.g. extract potency. Consequently, this project includes an "acclimated reproduction rate comparison".<sup>[47]</sup> In this study the response to light of different intensities by the different clones of *G. toxicus* is being monitored. Four light intensities are being used and include 80, 160, 205 and 250 footcandles of illumination. The reproduction rate (divisions per day) of the clones is plotted against the light intensity and the resulting slope of the line is used as a numerical systematic

character. It is critical that the cultures be completely acclimated in this study, as in the toxicity study. The slope of the line is a valid genetic character because the reproduction rate is under enzymatic control and varies with the efficiency of enzymatic transcription (hence chromosomal differences) under different environmental conditions. These data from these studies will also be useful as a data base in physiological studies which will examine the effect of light on toxin production.

Seventeen clones of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus* were physiologically adapted to the same environment over several months. There were significant variance components detected between non-acclimated and acclimated cells for the cell potencies, yields and reproduction rates of these cultures.

#### Toxin potencies

Outbred Harlan Sprague Dawley ICR(BR) mice weighing approximately 20 g were used to assess quantitative differences in the potencies of the methanol extracts. The potencies were then determined as LD<sub>50</sub>'s for each clone using from 3 to 10 mice at each of 4 dosage levels. The mice were observed for 48 h. The LD<sub>50</sub>'s were determined from the linear interpolation tables of Weil.<sup>[55]</sup> Alternatively, in some cases the LD<sub>50</sub> was determined by linear regression.<sup>[58]</sup> The final values are expressed as the number of mouse units (LD<sub>50</sub> dose for a 20 g mouse) per mg of dried cells, per cell of *G. toxicus* and in terms of number of cells per mouse unit.

The acclimated and non-acclimated cell potencies were compared in the same manner as the growth rate and cell yield statistics. In addition, a one-way Analysis of Variance with Replication<sup>[58]</sup> was used to compare selected clones from broad areas. The  $f_{\max}$  test<sup>[58]</sup> was used to assess homogeneity before conducting all ANOVA's. The potencies used in this ANOVA were plotted vs. latitude of collection along with the values for all other clones. The clones used in generating the ANOVA are also plotted with their 95% comparison intervals, calculated from and a-posteriori

T-method comparison among means.<sup>[58]</sup> Potency and latitude were also tested for correlation by the Pearson test.<sup>[58]</sup>

Three clones (177, 350 and 135) were also assayed for potency at 6 to 11 points during the growth cycle and these were plotted vs. days in culture and compared. The two parameters were then tested for correlation by the Pearson test.<sup>[58]</sup> For clone 177, 3 of 11 samples were assayed for potency 4x and compared by one-way ANOVA to test for significance of variation in potency through the acclimation process. The means from this test were also compared by the T-method. The potency and reproduction rate of clone 177 was also monitored after continuous batch culturing was stopped and transfers were made in stationary phase. Potency changes for clone 350 were monitored through acclimation to the vita lite bulbs and then when returned to a cool white light environment at a similar light intensity.

As a control experiment on sub-clone variability, clone 175 was sub-cloned 25x, all isolates survived and 4 were selected randomly to determine the coefficient of variation among fully acclimated sub-clones. As a control experiment on the stability of the acclimated condition, clone 135 is still being monitored for potency data accumulation beyond one year.

Five other relationships were also explored by correlation analysis and include potency vs. cell size and potency vs. reproduction rate for all clones examined. The former test was performed on acclimated potencies only whereas the latter test included all data points. Cell size was determined as the transdiameter and computed from a minimum of 20 cells of each clone collected from a log-phase culture. The potency was also tested for correlation with chlorophyll a, chlorophyll c<sub>2</sub> and peridinin content (pg cell<sup>-1</sup>). Pigments were assayed via the methods of Jeffrey et al.<sup>[53]</sup> and Indelicato and Watson.<sup>[59]</sup> They were subsequently extracted and analyzed for composition of proteins (Bio-Rad method), lipids<sup>[51,52]</sup> and carbohydrates.<sup>[49,50]</sup>

### Extraction of toxins

The initial methods for the extraction of the toxins is very similar to what is currently being utilized by other investigators (especially those attempting to isolate toxin from fish tissues) so that, to some extent, we can compare the toxic fractions which we obtain with what is in the literature. The cells are no longer extracted by refluxing in boiling aqueous methanol. Instead, extraction is now accomplished by crushing of cell, followed by sonication in methanol at room temperature. The methanol extracts are concentrated and subjected to liquid-liquid partitioning followed by cold acetone precipitation of the toxic components.

Further purification of the toxin will be either by thin layer chromatography (TLC), silicic acid chromatography or high pressure liquid chromatography (HPLC). Eluting solvents for column chromatography consist of chloroform-methanol (1:1), and chloroform-methanol-water mixtures (9:1:1). The eluting solvent for HPLC is methanol (100%).

### Purification of toxins from mass culture

Separation is achieved using three HPLC instruments associated with a single computer controller system. All three are Waters Company instrumentation and consists of a Delta 3000 Preparative-Semipreparative HPLC and a Model 300 Analytical system all interlinked with SIM modules to an 840 Controller System. While we use C-18 or C-8 columns in each system, the sizes differ, having 15 $\mu$  in the Preparative and Semipreparative and 10 $\mu$  or 5 $\mu$  in the Analytical.

### Mouse bioassay

The mouse bioassay is the officially recognized toxicity assay for ciguatera recommended by the Official Organization of Analytical Chemists and the FDA. In addition, it provides a base line against which we can compare our isolated preparation assays. The carrier for toxic extracts is normal saline containing 0.5 ml of a 1% Tween-60

solution. Toxicity is determined by an intraperitoneal injection of 0.5 ml of a suspension of extract into approximately 20 g mice (Strain CRE:CD:BR:ICR). Toxicity is defined as death of the mouse within 48 hours. LD<sub>50</sub> values are calculated according to the method of Weil.<sup>[55]</sup> Four dosage levels are used with three repetitions at each level. The LD<sub>50</sub> is calculated from moving average interpolation tables.

### Ileum assay procedures

We have utilized the terminal portion of the guinea pig ileum to assay dinoflagellate toxins. The overall setup for the ileum assay is shown in Figure 2.

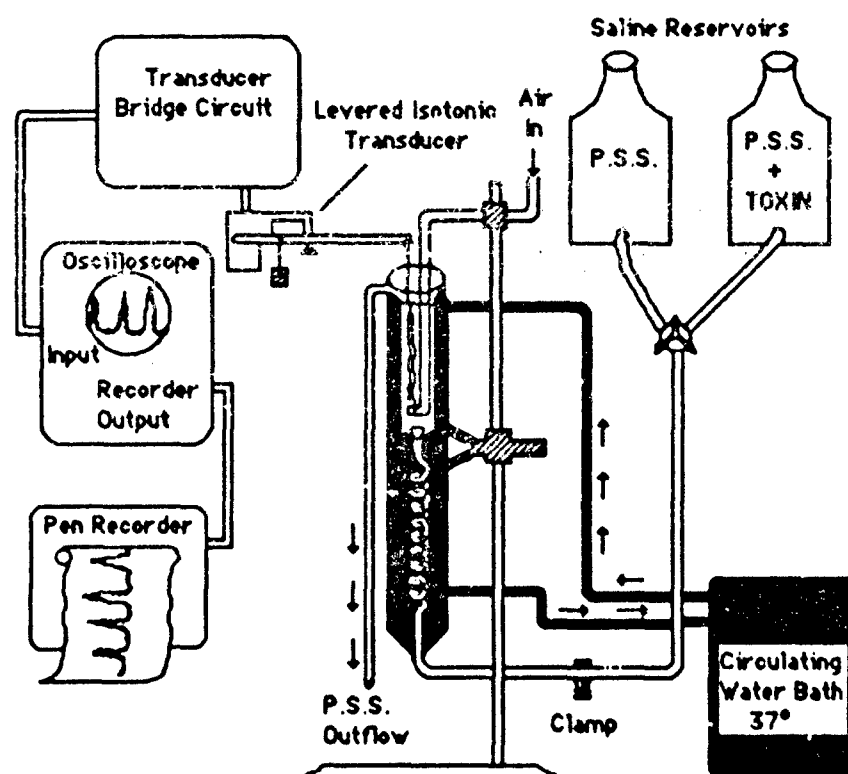


Figure 2. Schematic diagram of the equipment setup for the guinea pig ileum preparation. Individual components are discussed in the textual material.



**Guinea Pig Ileum.** Female guinea pigs (350-600 g) were sacrificed by a cervical dislocation. A 2-4 cm segment of the terminal ileum was removed and placed in physiological saline solution (PSS) at 37°C. The terminal portion will, however, respond to exogenously applied agonists. Hence its suitability for use in an assay system.

**Physiological Saline Solution.** The physiological saline solution for the guinea pig ileum consists of the following (mM):

TABLE I  
PHYSIOLOGICAL SALINE COMPONENTS

CHEMICAL	mM	g/L	Stock g/L	amt/L
NaCl	136.9	7.014		weigh out
KCl	2.68	0.372	186.37	2.0 ml
CaCl <sub>2</sub>	11.84	0.277	55.5	5.0 ml
MgCl <sub>2</sub>	1.03	0.095	23.8	4.0 ml
NaHCO <sub>3</sub>	11.9	0.084	8.4	10.0 ml
KH <sub>2</sub> PO <sub>4</sub>	0.45	0.178	89.1	2.0 ml
glucose	5.55	1.980		weigh out

**Tissue Bath.** The excised terminal ileum is allowed to come to temperature in saline for 15 min then a one cm segment is cut and inserted in an Anderson type tissue chamber<sup>[60]</sup> modified as suggested by Bartelstone (Figure 2).

**Water Circulating Bath.** The guinea pig ileum is extremely temperature sensitive and changes as small as 0.3°C may affect its contractility and therefore your results. For this reason you must have a circulating water bath or some other means of controlling the temperature. The circulating water bath that we presently use is Fisher Model M8000, Isotemp, Constant Temperature Circulator. The ileum is connected to a locally-made device which is a true, isotonic-tension transducer. The counter weight we use is 2 grams. Amplification of the transducer signal is achieved by the use of a DC oscilloscope with a pen output. For storage of records and recording of data for calculations we chose an inexpensive chart recorder (Fisher Recordall Model 5000). Any good DC level recorder can be used, but a ten inch recorder makes the task of measurement much

easier. The ileum has been shown to respond to acetylcholine, histamine, substance P, epinephrine, and several other compounds. Indeed, it has varying sensitivity to each of these compounds. We have found that the toxic fractions will give different inhibitions with different agonists.

**Protocol for Reversible Toxins.** The first protocol followed is utilized when there is only a reversible toxin in the extract. Initially a control series of acetylcholine or histamine stimulations is performed at different dosages to determine three doses which would give us values between 20 and 80% of maximum contraction of the ileum segment (Figure 3). That particular set of test doses is then utilized throughout the rest of the experiment. All subsequent responses of the ileum to agonist challenge are expressed as a percent of control values.

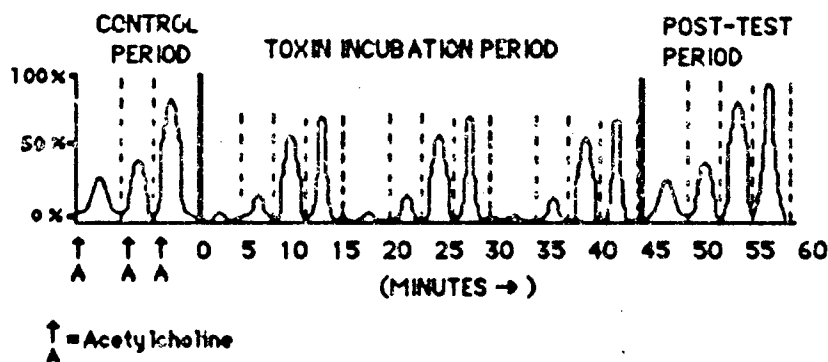


Figure 3. Schematic diagram of the methodology for the assay of a reversible toxin.

The toxin containing physiological saline solution is utilized for subsequent washes. Thus when testing for reversible inhibition, the toxin is always present in the PSS at the same concentration.

**Non-reversible Assay.** The second protocol is utilized when it is suspected that there only a non-reversible toxin in the extract. First a control series of histamine stimulations is performed at different dosages to determine a dose which would give us approximately 80% of maximum contraction of the ileum segment

(Figure 4). That particular test dose is then utilized throughout the rest of the experiment. All subsequent responses to histamine are expressed as a percent of that control value.

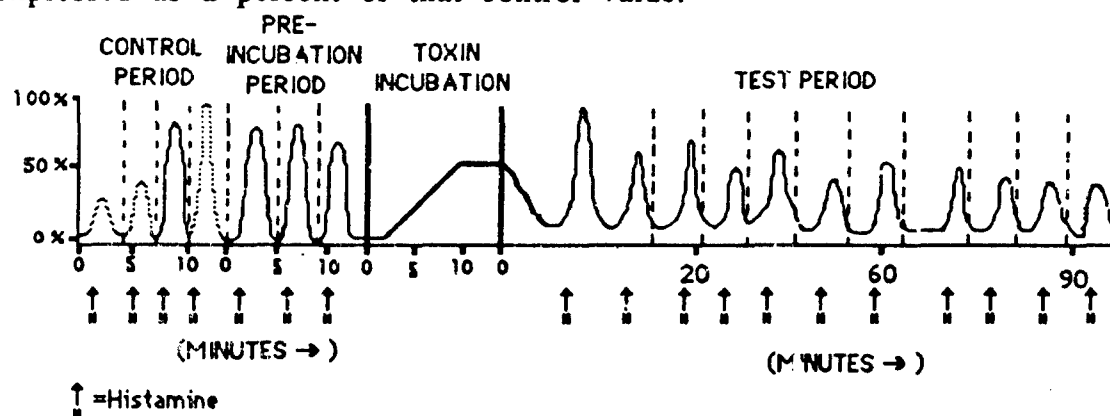


Figure 4. Schematic for the analysis of an irreversible toxin using the guinea pig ileum.

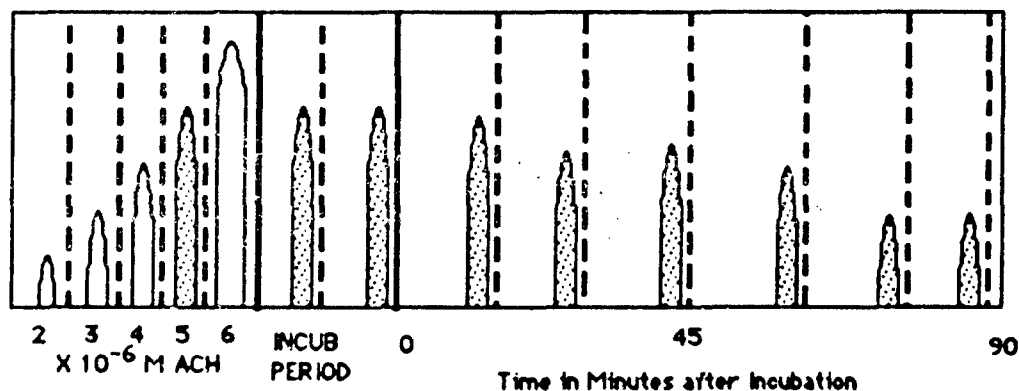


Figure 5. Schematic diagram illustrating methodology for using the ileum assay when both an irreversible toxin and a reversible toxin is present.

### Tests for purity of toxins

The easiest manner to test for purity of the toxin is to run in a recycle mode on the analytical HPLC to determine if after a given number of recycles the single peak remains or resolves into more than one peak. We will use several criteria for testing for purity: first the presence of single peak after recycling on analytical HPLC;

second, migration on TLC plates in different solvent systems, and structural data from Nuclear Magnetic Resonance.

#### NMR spectroscopy

At the present time Nuclear Magnetic Resonance (NMR) would provide us with the most informative data and yet is non-destructive to the sample analyzed. For these reasons it is the method of choice. Samples of purified toxins will be sealed in special, thin-walled, small-bore, NMR sample tubes from Wilmad Glass Co. Two state of the art NMR instruments are presently available for our use: specifically a 300 MHz Varian VXR-300 and a 500 MHz Varian VXR-500 multinuclear spectrometer system. Both instruments operate in the pulse Fourier Transform mode and are equipped with a liquid helium VXR superconducting magnet and acquisition hardware. Both have H<sub>2</sub> fixed frequency lock system with 5 mm broad band computer switchable probe. Multiple probes to two chambers accepting, 5 mm (narrow bore) and 10 mm (medium-wide bore) tubes for <sup>1</sup>H, <sup>13</sup>C, <sup>31</sup>P, <sup>15</sup>N, <sup>19</sup>F and other nuclei. Quadrature detection. Homo- and hetero-nuclear decoupling with spectral limits of 100 to 100,000 Hz. Variable temperature control (-70° to 140°) under computer control for all probes. VXR data station with dual high density disks. The VXR-300 operates at 300 MHz <sup>1</sup>H resonance and is presently used primarily for <sup>13</sup>C analysis. Automatic performance of standard relaxation experiments, as well as data recording is accomplished by an associated computer. The chemical shifts recorded are then interpreted for structure.

#### Packaging of toxins for delivery

Purified toxins in 100% methanol will be placed into vials, concentrated to almost dryness under nitrogen gas, sealed and labeled for shipment. The vial is then encased in a plastic container with absorbant material. The plastic container is then packed into a metal container and sealed. This is enclosed in packing material and styrafoam box surrounded by a cardboard container. The entire package is shipped by overnight express.

## RESULTS

### Acquisition of toxic dinoflagellates

Since the inception of our work on Ciguatera in 1978 we have conducted an extensive survey of ciguatera endemic regions of the British and United States Virgin Islands which resulted in the collection of 46 species of dinoflagellates. Sixty-five strains representing 18 of the most conspicuous epiphytic, benthic and planktonic species were isolated and brought into unialgal culture, harvested, and subjected to our standard extraction procedures. We proposed to continue our yearly survey for toxic dinoflagellates.

### Overall objectives for the third year

Our third years objectives can be summarized as follows:

- (1) Continuation of mass culture of *Gambierdiscus toxicus* and initiate culture of *Ostreopsis*.
- (2) Continue biosystematic studies to improve toxin production.
- (3) Extraction of crude toxins from mass cultures.
- (4) Bioassays of isolated extracts.
- (5) Purification of toxic fractions.
  - a. Continue to refine GT-4 purification
  - b. Initiate purification of GT-1
- (6) Investigation of factors affecting the stability of stored toxins.
- (7) Quantitation of toxins by weight and bioassay.
- (8) Examination of toxins by NMR.
- (9) Delivery of toxic fractions to U. S. Army Medical Research Institute of Infectious Diseases.
- (10) Continue to search for additional toxic dinoflagellates for additions to the SIU culture collection.
- (11) Maintain the SIU culture collection.

### Maximizing Toxin Production in *Gambierdiscus toxicus*

Cultures were grown at five light intensities at 28°C, at six light intensities at 25°C, and limited growth occurred at 22°C. The experiment is still continuing with replicates now being grown at

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higher light intensities at 25°C. We initiated two new light intensities at 25°C (3782 and 4800 lux) because growth did still not appear to be light-saturated at 28°C at 3350 lux (Fig. 6). The 3782 lux light intensity also failed to foster complete saturation and the 4800 culture is now growing. Thus, an even brighter light intensity was used when we moved to 22°C, but cultures only continued at maintenance levels at this temperature. This light/growth pattern is unusual for *G. toxicus* as most clones of this species cannot grow beyond 3,000 lux (pers. obs.). Growth could not be sustained at 22°C at the two lowest or two highest light intensities used. We conclude that clone 175 is more light tolerant and less temperature tolerant than other clones of *G. toxicus* based on comparisons to clones used in previous work.<sup>[61]</sup>

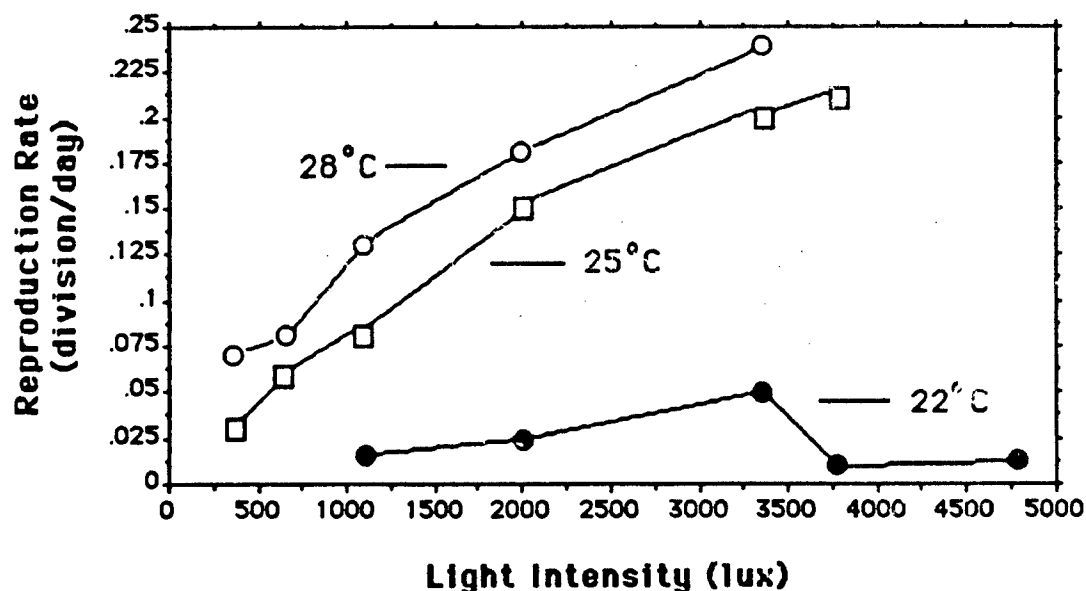


Figure 6. Reproduction rate (divisions/day) of clone 175 vs. light intensity. The pattern is shown for growth at 22, 25 and 28°C.

All cultures grown thus far at 25 and 28°C have been extracted and analyzed for protein content in duplicate (Fig. 7). Protein content, like most of the other molecular components examined, appears to follow a "normal" or bell-shaped distribution over the range of light intensities used (Fig. 8).

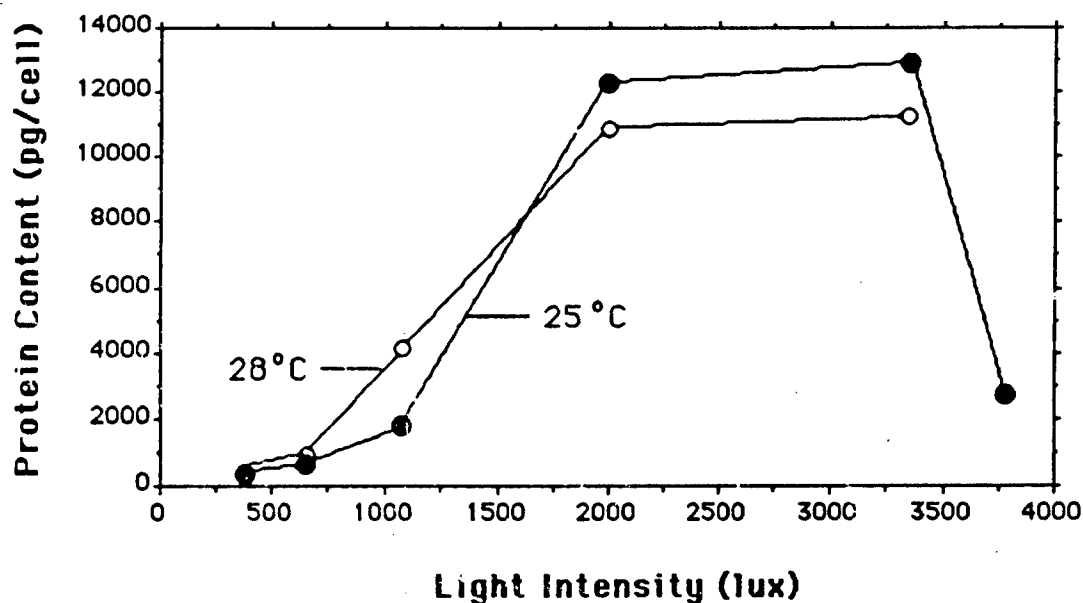


Figure 7. Protein content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 25 and 28°C.

Carbohydrate contents from 28°C are given in Fig. 8. Lipid content has also been examined at 28°C (Fig. 9). Carbohydrate production was greater in low light than in high light (Fig. 8). Lipid content closely paralleled chlorophyll production. The chlorophyll and all other pigment data presented here has been updated from the last report to include replicate analyses. The chlorophyll also shows increases with brighter light and then declines at the highest light intensities used (Fig.'s 8, 9 and 10). This pattern is of interest considering that growth rates continue to climb (Fig. 6) despite the obvious decay in the light-harvesting apparatus (decreased pigment contents). Chlorophyll production among the three temperatures was similar, again, showing the bell-shaped distributions. Cultures in the lower temperature produced less pigment (Fig.'s 9 and 10).

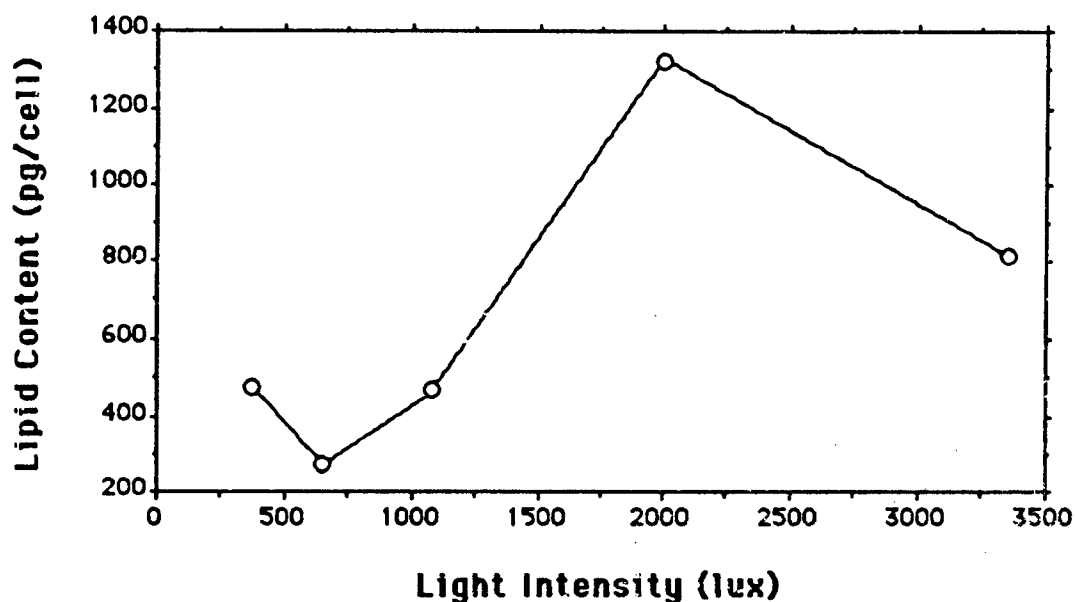


Figure 8. Lipid content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 28°C.

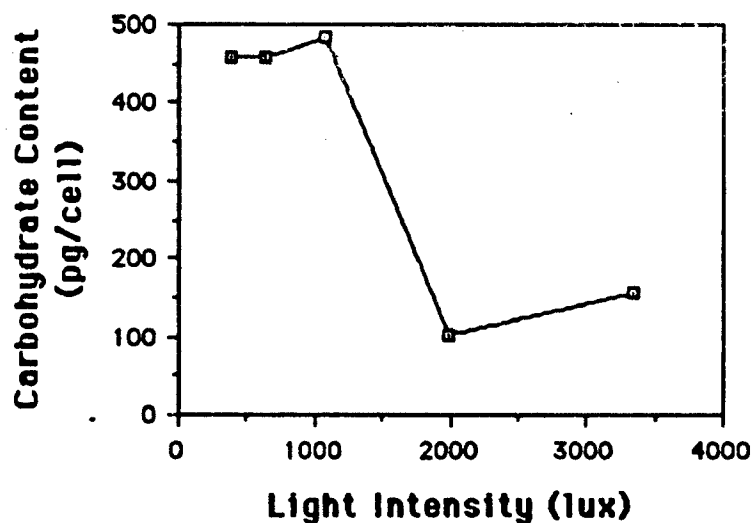


Figure 9. Carbohydrate content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 28°C.

The accessory carotenoids increased linearly with brighter light at the highest temperatures, and the carotenoids indicate that the cultures are obviously in decline at 25°C and only in maintenance at 22°C (Fig.'s 11, 12 and 13).



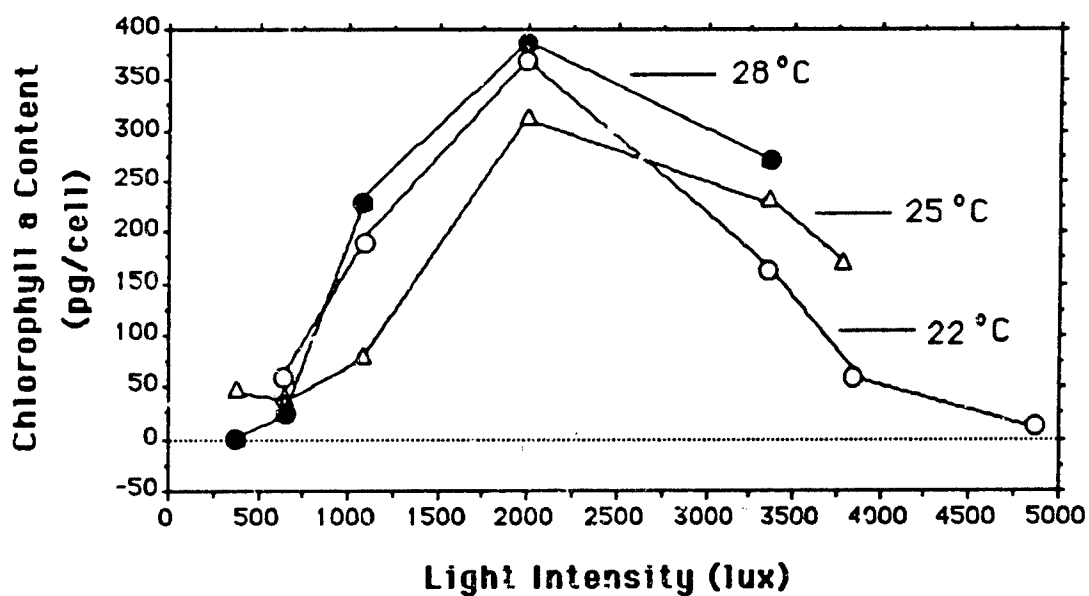


Figure 10. Chlorophyll a content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 22, 25 and 28°C.

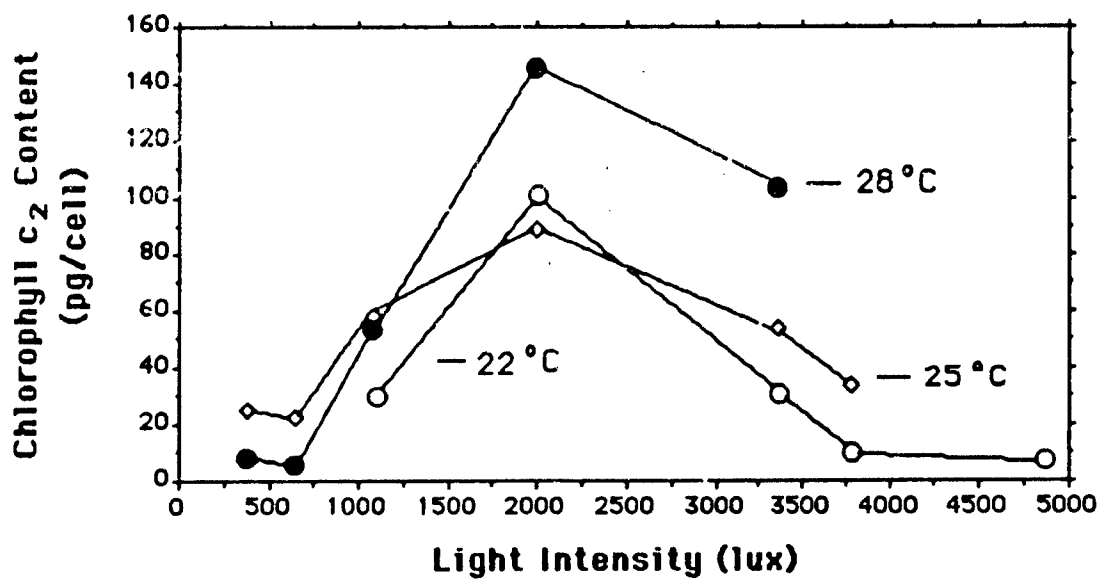


Figure 11. Chlorophyll c2 content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 22, 25 and 28°C.

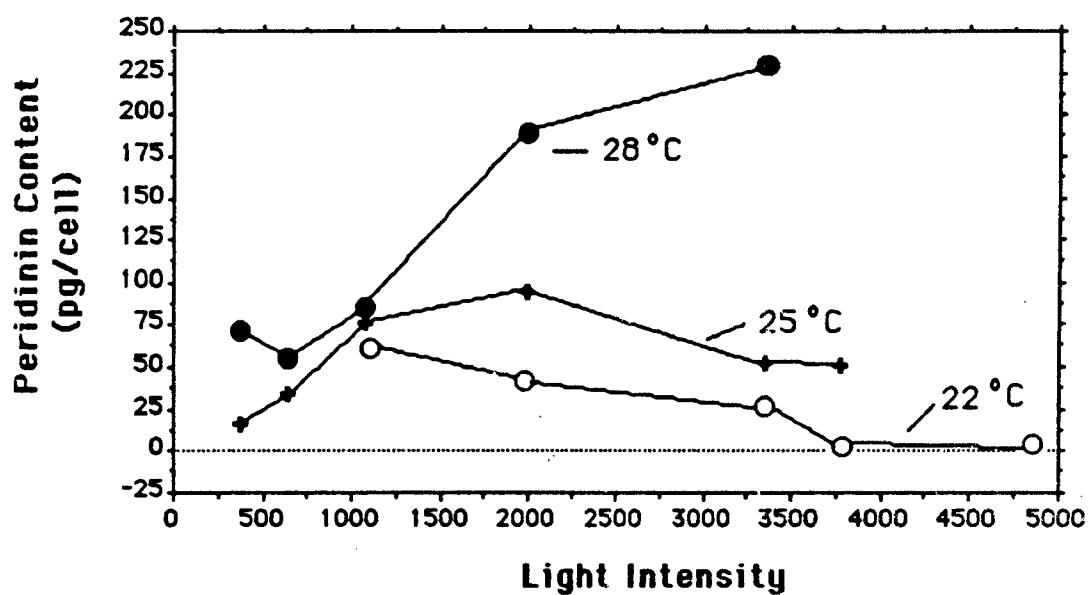


Figure 12. Peridinin content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 22, 25 and 28°C.

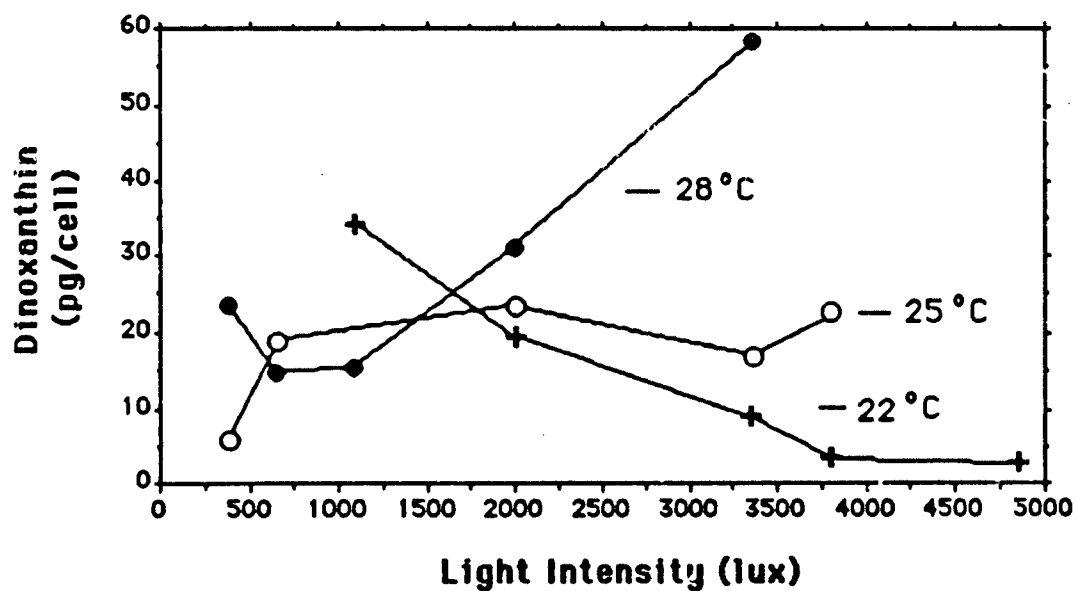


Figure 13. Dinoxanthin content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 22, 25 and 28°C.

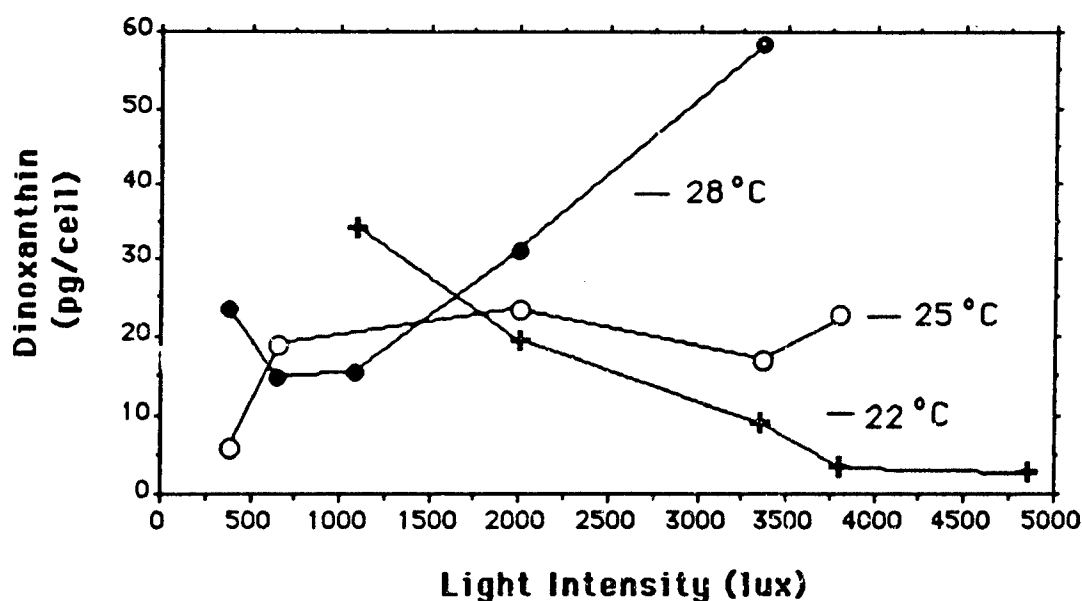


Figure 14 Diadinoxanthin content (pg/cell) of clone 175 vs light intensity. The pattern is shown for growth at 28°C.

Total toxin production has a normal, bell-shaped distribution over the range of light used at 22, 25 and 28°C (Fig. 14). It was exciting to learn that decreased temperatures improve toxin production substantially (x10). Experiments are now underway in which large cultures (10L) will be grown to appreciable biomass levels at 28°C and then subjected to temperature shock at 22°C. Hopefully, this will promote the increase in toxin production without sacrificing the reduction in biomass that is typical for cultures grown at 22°C. Toxin production was not well correlated with the production of any macromolecular components. There were slightly negative correlations between potencies and carotenoid production. However, the most interesting relationships were found between the culture potencies, population densities and ammonium uptake rates. The potencies are significantly and positively correlated with both of these parameters (Fig.'s 16 and 17).

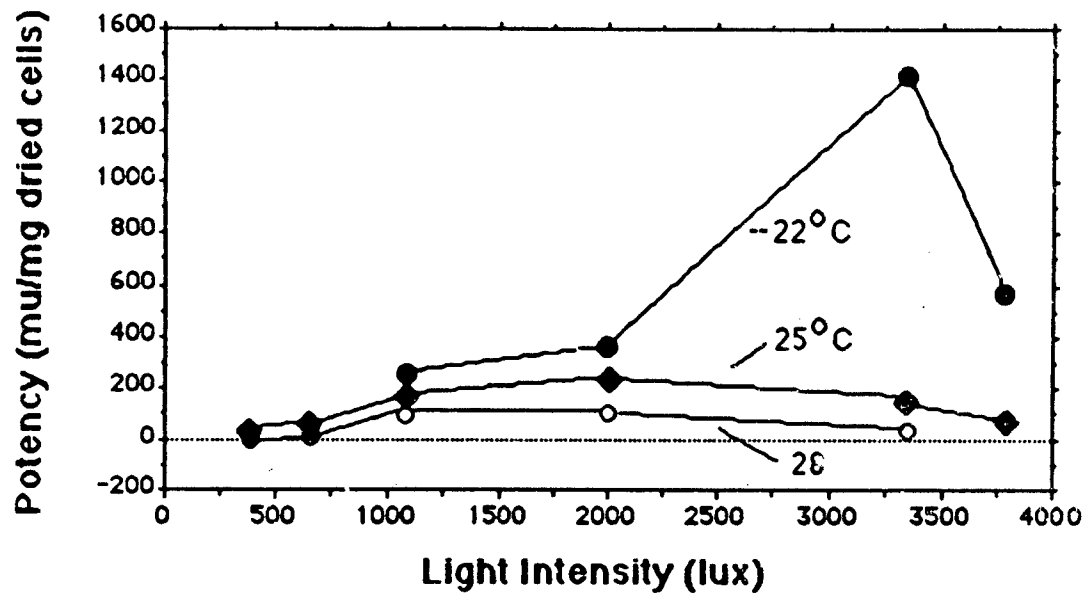


Figure 15. Culture potency (mu/mg dried cells) of clone 175 vs light intensity. The pattern is shown for growth at 22, 25 and 28°C.

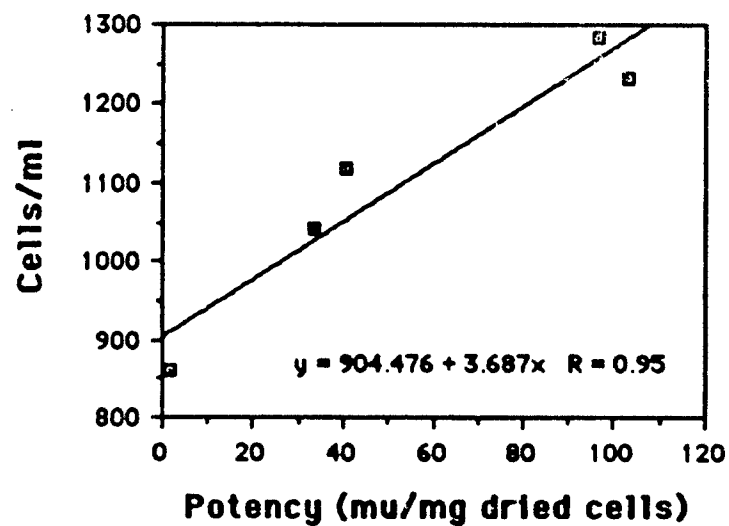


Figure 16. Culture population density (cells/ml) vs. the potency (mu/mg dried cells) of clone 175 (grown at 28°C).

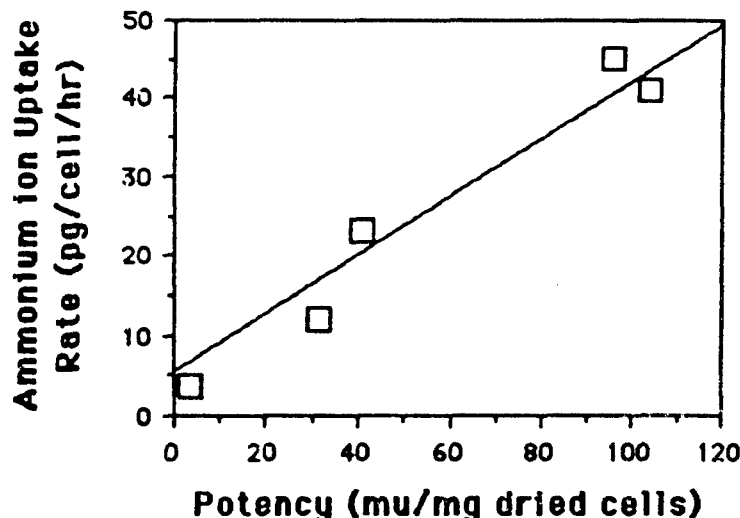


Figure 17. Ammonium uptake take rate (pg/cell/hr) vs. the potency (mu/mg dried cells) of clone 175 (grown at 28°C).

A correlation matrix has been generated on the data acquired thus far. That is, each component analyzed has been tested for association with all other components. The matrix is subject to change at this point because we are awaiting additional data from all three temperatures. The most significant relationships detected to date are given in Table 2.

TABLE 2.

SUMMARY OF THE CORRELATION MATRIX PERFORMED ON DEPENDENT DATA FROM *G. TOXICUS* GROWTH EXPERIMENTS.

Variable 1	Variable 2	Correlation Coefficient
Light Intensity	Reproduction Rate	0.99
Protein	Chlorophyll c	0.96
Protein	Peridinin	0.96
Potency	Ammonia	0.96
Potency	Cells/ml	0.95

As expected, light and reproduction rate are well correlated. Light intensity appears to have far greater effects on most parameters

compared to temperature. However, the effects of a temperature on toxicity is the major exception. Protein content was positively correlated with the three major pigments ( $r = 0.92$  to  $0.97$ ).

Large scale culturing of *Gambierdiscus toxicus*

TABLE 3.  
HARVESTS OF *G. TOXICUS*

CULTURE DESIG.*	DTE STARTED	VOL (ltrs)	YIELD GM		
			Wet Wt	Dry Wt	Tot Dry Wt
Material on hand from 1987 culturing					80.51
GT350-2A88D	25Jan	235	117	17.31	97.82
GT350-3A88D	12Feb	31	26.5	3.5	101.32
GT350-5A88D	6Apr	226	81.6	12.2	113.52
GT175-3A88	29Feb	216	80.0	10.0	123.52
GT175-4A88	2Mar	187	28.71	3.58	127.10
GT175-5A/88D	16Apr	230	162.1	30.6	157.70
GT350-7A/88D	8Jun	194	100.4	18.0	175.70
GT350-7B/88D	9Jul	184	112.9	21.1	196.80
GT175-8A/88D	31Jul	199	87.9	16.6	213.40
GT350-8A/88D	5Aug	197	87.99	16.68	230.08
GT350-10A/88D	9/20	190	94.4	17.7	247.78
GT350-11A/88D	10/25	193	81.5	13.2	260.98
GT175-9A/88D	9/6	186	128.5	19.3	280.28
GT175-11A/88D	10/11	168	159.9	26.44	306.72

TABLE 4  
GROWTH PRODUCTION DATA

	Used	Shipped	On Hand
Grams 350	(10.2)		190.00
Grams 175	(83.2)		9.74
Totals	93.4		213.32

The growth of clone 175 during this third contract year has produced in excess of forty million mouse units. The record of production is shown in Figure 18.

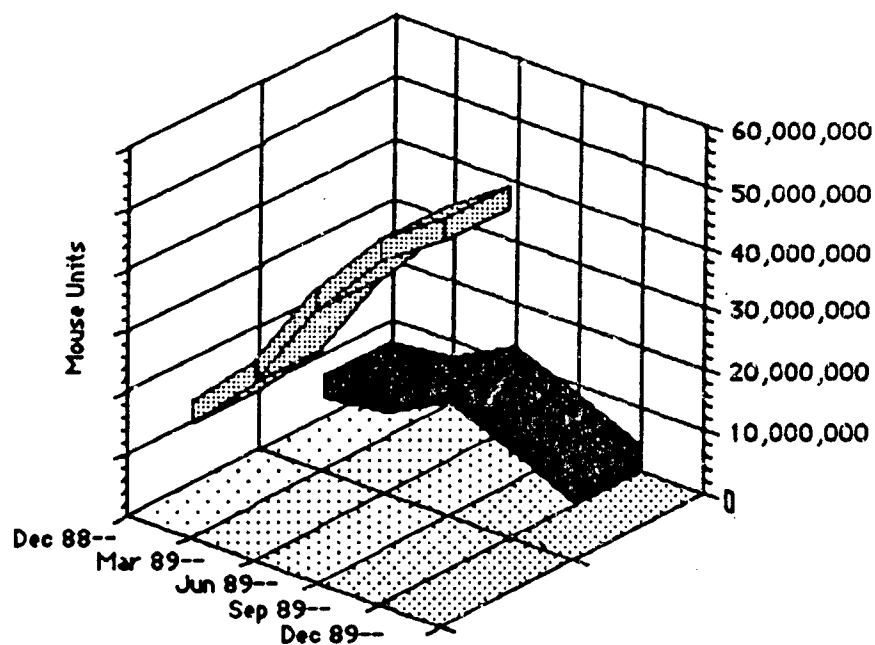


Figure 18. Mouse units produced per month and cumulative totals for contract year 1989.

During the third year of the contract the production of *Gambierdiscus toxicus* has been exceeding all expectations and projections. This has primarily been a result of our chemosystematic studies and switching from growing clone 350 to clone 175. The significance of this is appreciated when one examines the total estimated mouse units produced as shown in Figure 19.

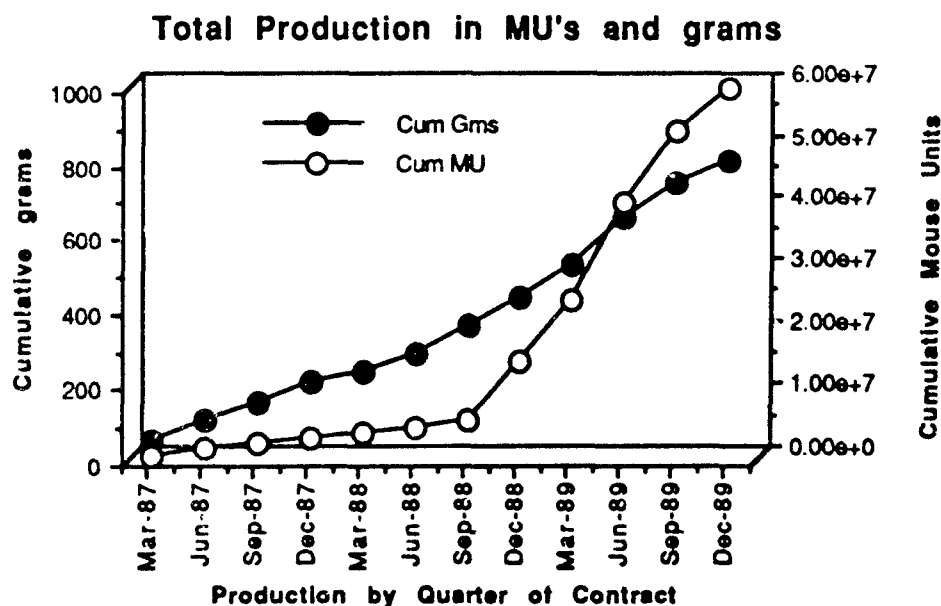


Figure 19. Plot of estimated mouse units and grams contained in cultures of *Gambierdiscus toxicus*, clones 350 and 175 listed by quarter for entire contract.

The mass culture of GT-175 was initiated in August of 1988. Notice the phenomenal increase in mouse unit accumulation. The switch to clone 175 has given us more than a ten fold increase in production capacity for GT-4 toxins. In addition, examination of the results from separation experiments indicate that the material from clone 175 has much less contaminants and therefore provides a much cleaner product for purification. Inasmuch as growth is no longer a problem for the production of GT-4 (maitotoxin?) we have now initiated additional chemosystematic studies to examine increases in the GT-1 (ciguatoxin?) fraction of *G. toxicus*.

#### Purification of Toxins from *Gambierdiscus toxicus* (Clone 175)

After comparing the results from several "first-step" treatments of crude methanol extracts from clone 175 we have concluded that small-scale liquid-liquid partitioning yields the highest quantity and quality of toxic products. An example of such a separation and results thereof are shown in Fig. 20. Without consideration of the weakly toxic ESAF fraction, we consistently recover over 80% of the total MU's in the more potent ESMF and WSMF fractions. Methods for large-scale purification of ESMF and



WSMF (from clone 175) developed include: (1) acetonitrile precipitation and (2) low pressure semi-preparative C-18 reverse phase chromatography.

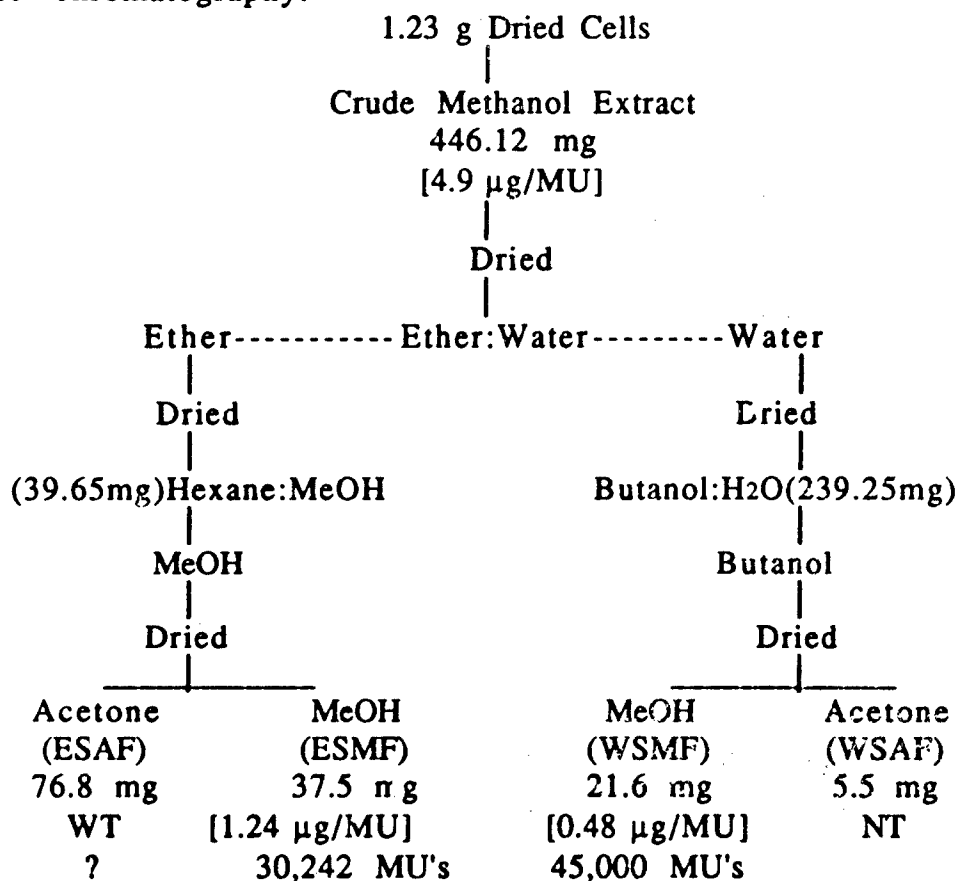


Figure 20. Results from liquid-liquid partitioning of a crude methanol extract from *G. toxicus* (clone 175).

Acetonitrile precipitation - WSMF in methanol was slowly added to acetonitrile to a final volume ratio of 2:8. The solution was allowed to stand at room temperature for 72 hours. During this period the solution was centrifuged at approximately 12 hour intervals. After 72 hours the precipitate was removed and assayed for potency using 20 g mice (some were assayed using the guinea pig ileum preparation). Five such treatments were completed. All supernatants displayed some degree of toxicity. These were consolidated and stored for future purification and assay. One sample of ESMF was subjected to the same treatment. Results from this purification step is shown in Fig. 21.

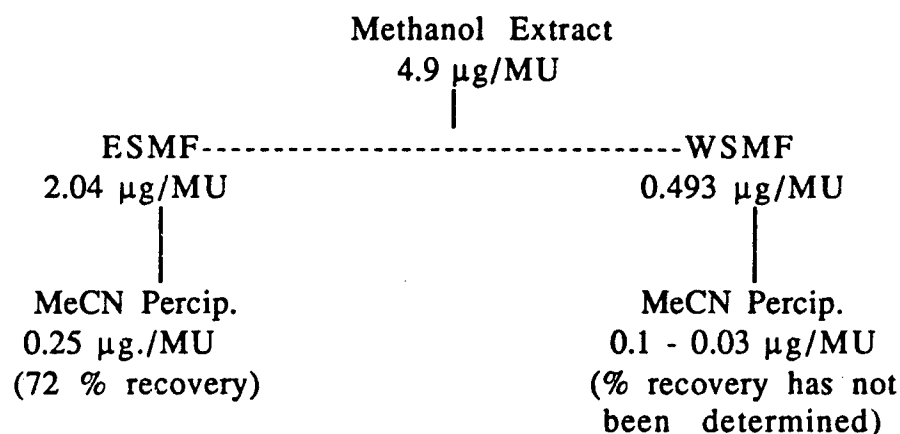


Figure 21. Results from one acetonitrile precipitation treatment.

Note that the acetonitrile treatment resulted in a 816.0% improvement in potency of ESMF and up to a 1643.3% improvement in potency of WSMF. Although this method currently yields somewhat variable results, one treatment yielded "MTX" with a potency of 0.03  $\mu\text{g}/\text{MU}$  (10% pure based upon Yasumoto's estimated MU of .003  $\mu\text{g}$ ). The LD<sub>50</sub> of our most potent fraction is 1.5  $\mu\text{g}/\text{Kg}$  mouse. This is the best purification of MTX reported from *G. toxicus* except for that of Yasumoto and co-workers. Results of assays of this toxin on the guinea pig ileum preparation are shown in Table 5

TABLE 5.  
ILEUM ASSAYS OF "MTX" (LD<sub>50</sub> 1.5  $\mu\text{g}/\text{KG}$  MOUSE)

Toxin in bath solution pg/ml	% Inhibition of ACH response	
	15 min.	90 min.
1.5	0.0	5.54
15.0	64.5	72.15
150.0	100.0	100.00

This is the most potent "MTX" fraction which we have assayed with the ileum preparation. The irreversible nature of this toxic fraction is consistent with previous reports for MTX. Also, these

results confirm our previously reported ratio of 2000 ileum units to one mouse unit (0.015 ng [ileum unit] X 2000 = 30 ng [mouse unit]).

TABLE 6.  
LOW PRESSURE LIQUID CHROMATOGRAPHY OF ESMF

Fraction	Solvent	Volume ml	Weight mg	Potency µg/MU	Total MU
1	Acetone	2.0	.06	NT	
2	Acetone	1.0	.39	NT	
3	Acetone	1.0	.96	.265	3623
4	Acetone	1.8	1.35	.9	1519
5	Acetone	2.0	.24	NT	
6	Acetone	15.0	.69	NT	
7	Acetone+	7.0	.45	NT	
8	Chloroform	14.0	4.14	6.0	690
	percip.		6.89	NT	
9	Chloroform+	5.0	.51	NT	
10	Methanol	2.0	.60	1.5	400
11	Methanol	4.0	.54	.9	600
12	Methanol	5.0	.18	NT	
13	Methanol	15.0	.30	NT	
14	Methanol	10.0	.15	NT	
TOTAL			17.45		6832 (82 %)

Low pressure C-18 chromatography -- Toxic products (ESMF and WSMF) from *Gambierdiscus toxicus* (clone 175) were subjected to low pressure liquid chromatography using a C-18 column (1.5 X 6.5 cm) and successive solvent applications including acetone, 95% chloroform:methanol, and methanol. Results from such a treatment of ESMF are shown in Table 6. The starting sample of ESMF (16.99 mg in 1 ml of methanol) was applied to a C-18 column which was preconditioned with acetone. The potency of the sample was 2.04 µg/MU (total 8328 MU)

Clearly, this first-step chromatographic treatment accomplishes considerable purification. For example, fraction 3 (.960 mg) displayed a potency of 0.265 µg/MU which represents a 769.8% increase in purity. More importantly, this procedure appears to have

resulted in the separation of the two different toxins expected in the ESMF fraction (fractions 3, 4, and 8 appear to be of the "CTX" type; whereas, fractions 10 and 11 appear to be of the "MTX" type). This procedure was used for separation of several additional ESMF fractions. Preliminary mouse assays revealed results similar to those shown in Table 6.

In an additional experiment eight grams of dried cells of *Gambierdiscus toxicus* (clone 175, harvest 9A/88D[2]) were extracted with methanol and yielded 2.56 grams of crude toxic extract. A total of 421.6 mg (100,024 mouse units) of this extract was subjected to liquid-liquid partitioning as shown in Figure 1.

Crude methanol extract and the six products of the partitioning procedure were assayed using mice. The WSMF fraction was dried under nitrogen and redissolved in methanol at which point crystals formed. These were immediately removed (slowly soluble in methanol and insoluble in acetone). Acetone was added to the WSMF solution until a precipitate formed. The mixture was centrifuged to remove the acetone insoluble precipitate. This precipitate was dried under nitrogen and brought up in about 1 ml of methanol and applied to a C18 column in two 0.5 ml lots. The column was developed with acetone under low pressure. The fractions collected and their approximate total volumes were 1A (3.0 ml), 1B (precipitate from 1A), 2 (5.0 ml), and 3 (37 ml). A final fraction, 4 (27 ml) was collected using methanol. Each fraction was assayed using mice. LD-50 determinations were completed using female mice (Harlan Sprague Dawley ICR 'BR') weighing 18-22 g. Doses of toxic fractions were suspended in 0.5 ml of 0.15M NaCl containing 1% Tween 60 and administered by intraperitoneal injection. Control mice were injected with carrier only. Four mice were injected with each of four doses of geometrically increasing concentrations. All mice were monitored for 48 hours. LD-50's were calculated using moving average interpolation tables provided by Weil.<sup>[55]</sup> One mouse unit (MU) is the LD-50 for individual 20 gram mice. Toxic fractions also were assayed using the guinea pig ileum preparation as previously described.

A summary of the results of the above treatment is shown in Table 7. Liquid-liquid partitioning yielded two toxic fractions (ESMF and WSMF) which accounted for 95.1% of the total mouse units present in the initial crude methanol extract. Treatment of the WSMF on the C18 column resulted in a single fraction (1B) containing 92.3% of the total mouse units present in the WSMF. A total of 99.4% of the beginning mouse units in the WSMF was accounted for in the various fractions. Fraction 1B contained 0.690 mg of toxin with an LD-50 of 0.7  $\mu\text{g/Kg}$  mouse (0.014  $\mu\text{g/MU}$ ). Based upon Yasumoto's estimation of the LD-50 of pure maitotoxin (0.15  $\mu\text{g/Kg}$  mouse), our Fraction 1B is approximately 25% pure.

The effects of this fraction on the ileum have been remarkable. For example, a concentration of 0.023 pg/ml of bathing solution caused 67.31% and 77.9% inhibition of the acetylcholine response in the ileum at 15 and 90 minutes (respectively) after incubation. A concentration of 0.0115 pg/ml caused 36.97% and 28.25% inhibition following the same procedure.

TABLE 7  
A SUMMARY OF THE TREATMENT OF 8 GRAMS OF DRIED CELLS OF *GAMBIERDISCUS TOXICUS* (CLONE 175, HARVEST 9A/88D12I) AND RESULTS AND ACCOUNTING OF TOXIN IN THE VARIOUS PURIFICATION STEPS.

FRACTION	TOTAL WT WT(mg)	POTENCY μg / M U	TOTAL MU	% RECOVERY
CELLS	8000.00			
MEOH EXTRACT	2561.63	4.215	600,232	
↓ MEOH EXTRACT RETAINED	1264.00	4.215	300,116	
↓ MEOH EXTRACT LIQUID-LIQUID PARTITIONED	1297.63	4.215	307,860	
↓ ESMF	421.60	4.215	100,024	
↓ WSMF	37.50	0.920	40,823	95.1
↓ CRYSTALS (1 & 2)	19.00	0.350	54,288	
↓ ACETONE SOLUBLE	0.60	0.700	857	7.1
↓ ACETONE PRECIPITATE	12.54	4.180	3,000	
↓ C18 COLUMN	5.86	0.116	50,431	99.4
↓ FRACTION 1A	1.14	1.500	760	
↓ FRACTION 1B*	0.69	0.014	48,936	92.3
↓ FRACTION 2	0.60	1.500	400	
↓ FRACTION 3	0.06	NT	---	
↓ FRACTION 4	1.62	NT	---	

\*Delivered to Dr. Judy Pace (621 μg in 3 ml, #0001BR)

Experience with this method of separation has shown that the most efficient amount to work with in the liquid-liquid extraction process is 2 grams of crude. Amounts larger than this seem to lead

to increased loss of toxin. The reason for this is not apparent at this time. Therefore, owing to the fact that the liquid-liquid separation is done 2 grams at a time, separation of large quantities takes longer.

A total of 13 g of dried *Gambierdiscus toxicus* (175, harvests 9A88D, 11A88D, and 3B89D) was extracted for the purpose of purifying GT-4 (MTX). Four g were expended in attempts to improve our purification procedure. We were successful in collecting a significant amount of relatively pure MTX from the remaining 7 g of cells. Extraction and purification of an additional 2 g has been initiated. A summary of the products of this purification are included in Table 3.

When the total 9 g has been taken through our preliminary purification series, we will have accumulated over 21.24 mg of MTX with a purity of 0.03-0.07  $\mu\text{g}/\text{mouse unit}$ . This represents a total of more than 362,904 mouse units. Assuming a mouse unit equivalent for pure MTX to be 0.003  $\mu\text{g}$  (based upon Yasumoto's best estimate), our total collection of 21.24 mg (5.1% pure) contains 1.09 mg of MTX.

You will note in Table 7 that there was a significant amount of toxin which partitioned to ether and ended up in the ESMF fractions. We believe that this fraction contains MTX or a derivative thereof. However, it may contain quantities of ciguatoxin (CTX) as reported by other researchers. We are developing new methodology which will help us determine the presence and quantities of multiple toxins in the ESMF fractions.

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TABLE 8.  
PRODUCTS OF EXTRACTION AND PURIFICATION OF MAITOTOXIN FROM *GAMBIERDISCUS TOXICUS*  
(175).

HARVEST	FRACTION	TOTAL WEIGHT mg	µg/MU	TOTAL MIJ's
11A88D 1B	DRIED CELLS	1,000.00		
	MEOH EXTRACT	307.50	2.49	148,795
	HEXANE	48.00	NT	NT
	ESAF	51.50	NT	NT
	ESMF	29.00	0.60	48,333
	WATER	222.60	NT	NT
	WSAF	NA		
	WSMF (MTX)	30.00	0.40	75,000
	C18-2B (MTX)	1.68	0.03	56,000
	C18-3	23.04	1.40	16,457
	C18-4	2.16	NA	NA
3B89D 1AB	DRIED CELLS	2,000.00		
	MEOH EXTRACT	645.00	2.346	274,936
	HEXANE	92.00	NT	NT
	ESAF	119.50	NT	NT
	ESMF	62.00	0.60	103,333
	WATER	317.52	NT	NT
	WSAF	1.56	NT	NT
	WSMF (MTX)	33.75	0.27	125,119
	C18-2B (MTX)	5.06	0.069	73,000
	C18-3B & 4 (MTX)	0.60	0.07	8,571
	C18-3A	17.16	2.86	< 6,000
3B89D 2AB	DRIED CELLS	2,000.00		
	MEOH EXTRACT	667.00	2.30	290,000
	HEXANE	93.25	NT	NT
	ESAF	122.00	NT	NT
	ESMF	72.50	0.70	103,571
	WATER	316.79	NT	NT
	WSAF	2.88	NT	NT
	WSMF (MTX)	35.72	0.236-.365	124,469
	PRECIPITATE (MTX)	4.40	0.063-.069	67,000
	SOLUBLE	12.66	2.00	6,342



TABLE 8. CONTINUED

3B89D 3AB	DRIED CELLS	2,000.00		
	MEOH EXTRACT	669.00	< 3.33	> 200,900
	HEXANE	89.60	NT	NT
	ESAF	119.50	NT	NT
	ESMF	57.00	0.60	95,000
	WATER	310.69	NT	NT
	WSAF	0.96	NT	NT
	WSMF (MTX)	33.08	0.279-.357	104,569
	IN PROGRESS (EST. MTX)	> 4.50	0.06	> 75,000
3B89D 4AB	DRIED CELLS	2,000.00		
	MEOH EXTRACT	652.50	2.23	292,600
	IN PROGRESS (EST. MTX)	5.00	< 0.06	> 83,333

### Separation of GT350 Material

In the processing of GT-350 samples, a radical approach to separation was initiated. Entire cultures were extracted with methanol and subjected to solid phase extraction. Each extraction was monitored by analytical HPLC (230, 330, 440 nm) and toxin level monitored by ileum assay. At each step we have attempted to selectively extract a pigment or class of compounds and keep the toxin precipitated. Previous experiments have shown that the toxic activity can be retained under these conditions.

Accordingly, 12.21 grams of dried cells (*G. toxicus* 350, culture 5A/88D) identified as 1000 series was processed to a methanol extract by sonication and methanol extraction as previously described. This crude extract was treated with hexane:methanol (1:1). Both the hexane fraction and the methanol fraction were assayed by ileum and the data is as follows:

TABLE 9  
HEXANE:METHANOL SEPARATION OF GT350, 1000 SERIES

Code	Fract.	Color	$\frac{\text{MU}}{\mu\text{g}}$	$\frac{\mu\text{g}}{\text{MU}}$	Total MU	%Rec	%Pur
1000y	Crude	Grn-Brn	.0039	254	48000	100	.0011
1000v	Hex Ext	Green			<5.0	0	-
1000g	Hex Ppt	Choc.Br	.1517	6.59	44000	91.6	.0455

As can be seen the hexane:methanol step was a very successful innovation in that almost all of the mouse units were retained but the weight was reduced such that the  $\mu\text{g}$  per MU value went from 254 to 6.59 and at the same time the % purity went up by more than a factor of ten. The second step involving the acetonitrile extraction was instituted on the fraction 1000g and the data is as follows:

TABLE 10  
ACETONITRILE EXTRACTION OF GT350, 1000 SERIES

Code	Fract.	Color	$\frac{\text{MU}}{\mu\text{g}}$	$\frac{\mu\text{g}}{\text{MU}}$	Total MU	%Rec	%Pur
1000g	Hex Ppt	Choc.Br	.1517	6.59	44000	91.6	.0455
1000w	ACN Ext	Red Brn	-	-	100	-	-
1000c	ACN Ppt	Pea Grn	0.588	1.70	38000	79.1	.1764

The acetonitrile extraction step has also proven to be very effective in extraction of peridinin and monopolar carotenoids meanwhile retaining toxic activity. With this step we achieve a purity level of .176% (based upon Yasumoto's figure of .003  $\mu\text{g}/\text{MU}$ ) and a specific activity of 1.7 $\mu\text{g}/\text{MU}$ .

The third step was one in which we attempted to isolate the toxic moieties from the large amount of chlorophyll c which remained. In this step a simple dilution-precipitation step using ethanol as the diluent for the methanol was instituted. A precipitate was obtained from the solution as follows:

TABLE 11  
ETHANOL PRECIPITATION OF GT350, 1000 SERIES

Code	Fract.	Color	$\frac{\text{MU}}{\mu\text{g}}$	$\frac{\mu\text{g}}{\text{MU}}$	Total MU	%Rec	%Pur
1000c	ACN Ppt	Pea Grn	0.588	1.70	38000	79.1	.1764
1000n	EtOH sol	Green	-	-	200	-	-
1000p	EtOH Ins	Green	0.95	1.05	36000	75.0	.285

This ethanol precipitation step is one which gets us to the 1  $\mu\text{g}/\text{MU}$  range or at about .3% purity. Thus, we are now able to achieve the 1  $\mu\text{g}/\text{MU}$  range of purification for GT350 very easily using large samples.

At this point it was decided that the preparative HPLC unit would be used for further separation of the toxin. Because an HPLC pilot study had shown that the composite peak at approximately 3.7 minutes elution time corresponded with toxicity, the entire sample of 36000 MU was applied to the preparative HPLC and the area corresponding the the 3.7 minute peak collected separately. Ileum assay of the fractions indicated that the large peak had 20,000 MU remaining. Analytical HPLC chromatograms indicated each preparative fraction had multiple peaks and none corresponded to toxin activity.

TABLE 12  
PREPARATIVE HPLC SEPARATION OF GT350, 1000 SERIES

Code	Fract.	Color	$\frac{\text{MU}}{\mu\text{g}}$	$\frac{\mu\text{g}}{\text{MU}}$	Total MU	%Rec	%Pur
1000s10	-3.0m	Clear	-	-	-	-	-
1000s2	-4.0m	Cream	3.93	0.285	20,000	41.6	1.2
1000s3	-6.0m	Clear	-	-	-	-	-

A different approach was taken on the remaining 20,000 MU's of series 1000 in that an acetonitrile precipitation was attempted to precipitate the toxin. Several subsequent steps were tried after the acetonitrile precipitation. However, these steps were not so successful for the retention of the toxin. Accordingly, a second 18.0

grams of dried cells (*G. toxicus* 350, culture 7A/88D) identified as the 3000 series was processed by the first two steps.

TABLE 13  
HEXANE:METHANOL SEPARATION OF GT350, 3000 SERIES

Code	Fract.	Color	$\frac{\text{MU}}{\mu\text{g}}$	$\frac{\mu\text{g}}{\text{MU}}$	Total MU	%Rec	%Pur
3000y	Crude	Grn-Brn	.0526	19	278000	100	.0157
3000g	Hex Pt	Choc.Brn	.0310	16.02	256000	92.0	.0156
3000c	ACN Pt	Pea Grn	0.087	7.8	250000	72.4	.026

From our previous small scale studies it was evident that the large concentrations of pigments and lipids interfered with HPLC separation of the toxic components in GT350. Therefore, we continued adding steps in the extraction scheme designed to eliminate the pigments entirely. To accomplish this, the first extraction was with hexane:methanol (1:1). This previously mentioned extraction step was expected to remove nonpolar carotenes and chlorophyll(s) a. During this step we discovered an unusual type of chlorophyll a contained in the dinoflagellate.<sup>[62]</sup> Once this step was accomplished a second solid phase extraction was accomplished utilizing acetonitrile. The step with acetonitrile was expected to remove peridinin and other monopolar carotenes. A third step was incorporated to selectively precipitate the toxic components out of solution and leave the remainder of the chlorophyll(s) c in solution.

The results have been impressive in that routine purification of very large quantities down to the level of 1  $\mu\text{g}/\text{MU}$  with retention of 95% of the toxic activity is now routine. The loss of toxic activity now comes with solubility and attempts to purify below this level. Separation of this material on a C18 column with methanol and water is producing large quantities (60,000 MU) per batch of 10% purity as determined by ileum assay.

The separation of 350 material by solid phase extraction as previously described has allowed purification of material down to

the 1% level with retention of 60% of the toxic activity (actually 175,000 MU). Inasmuch as the toxin content of 350 is much less and the amount of interfering lipids is higher than that of 175 much more material must be processed to accomplish the same purification.

We have recently acquired a viable culture of *G. toxicus* from the French Polynesian Islands which has been reported by Yasumoto, LaGrand, and others have reported to produce CTX. If the production of CTX is confirmed in any of our numerous clones of *G. toxicus*, we will initiate mass production of said clones for the specific purpose of collecting and purifying CTX.

#### Brevitoxin Antibody Competition Experiments:

Antibodies to brevitoxin were received from Dr. Mark Poli, USAMRIID and immediately put them to use. The procedure was to take a dose of toxin which we had calculated from previous runs would produce a 50% inhibition on the ileum. This sample of toxin would be combined with an aliquot of brevitoxin antibodies, allowed to incubate for 10-15 minutes and then applied to the ileum. The toxin + antibody was allowed to incubate for the regular 15 minute period and then the preparation was rinsed and challenged with the same control doses of agonist. To calculate the percent inhibition we add the heights of all the dose responses and divide by the number of doses for both the control and the experimentals - then calculate the percentage inhibition versus the pre-incubation control.

We started with a 100 fold dilution of antibody material added to GT-4 extract. No effect was seen so we used a 10 fold dilution. No effect was seen at this concentration so we utilized a 1 to 1 concentration. The results are shown in the Figure 22.

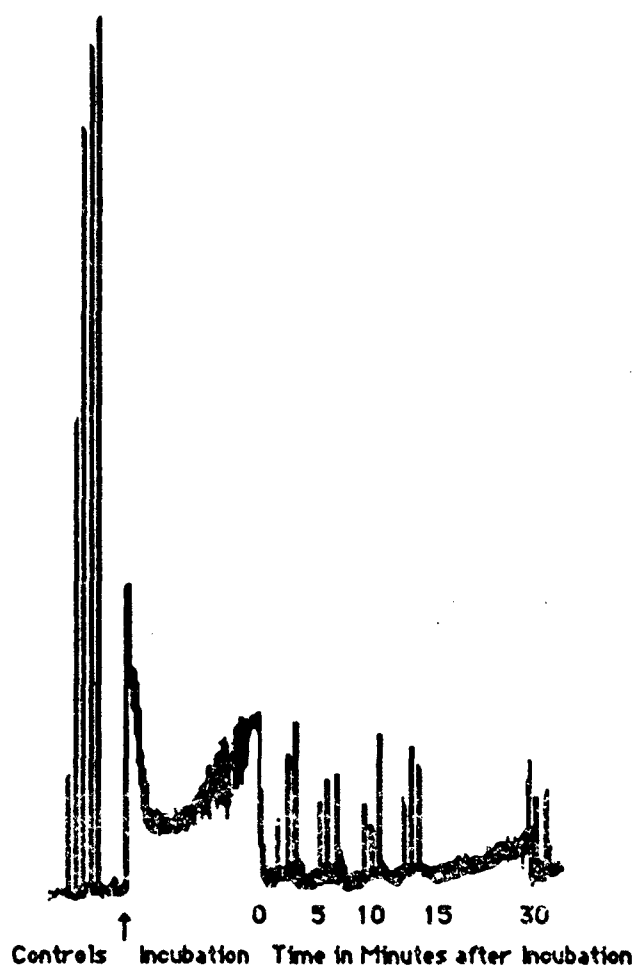


Figure 22. Controls were acetylcholine ( $1 \times 10^{-6}$ ,  $3 \times 10^{-6}$ ,  $5 \times 10^{-6}$ ,  $7 \times 10^{-6}$ , and  $9 \times 10^{-6}$ ). A  $5 \mu\text{l}$  aliquot of a 1:10,000 dilution of GT-4 was added to  $1 \mu\text{l}$  of anti-brevitoxin preparation, incubated for 10 minutes and applied to ileum preparation at arrow. After a 15 minute incubation the preparation was inhibited 87%.

It is quite apparent that the antibodies had no effect on the efficacy of GT-4 ("MTX") on the ileum preparation. Next, inasmuch as we had some fish toxin in storage that was provided by Dr. Michael Capra (Queensland Institute of Technology) we utilized the same procedure to test the efficacy of the anti-brevitoxin preparation against it.

Again the conclusion was that the antibodies had no protective effect for the ileum when toxic fish extracts were applied. Finally, we utilized an aliquot of GT-1 ("CTX") prepared from *Gambierdiscus toxicus* to see if in fact it would react.

We have repeated these experiments several times without results. I think that the conclusions right now are that, at least with the ileum preparation, brevitoxin antibodies provide no protection from either GT-4, GT-1 nor toxin isolated from toxic Australian fish. We have provided Dr. Poli with samples of the same toxins we utilized (GT350 GT-1; GT350 GT-4; GT175 GT-1; and GT175 GT-4) to see if they work better on his system.

#### NMR Studies of Purified GT-4 from GT-350

Recently we have run NMR spectra on two samples of high MTX content. NMR MTX #1 had 100,000 mu at a purity level of 300 ng/mu (1%) and was subsequently incorporated into a larger sample NMR MTX #2; 330,000 mu at a purity level of 30 ng/mu (10%). This further purification was achieved by washing the toxin with H<sub>2</sub>O on a C-18 column.

Large low field peaks at  $\delta 8.5$ ,  $\delta 8.12$  and  $\delta 6.92$  were removed and the whole spectrum profile was much reduced. Certain peaks in the  $\delta 7.5$  to  $\delta 5.0$  range appeared enhanced, although characteristic long relaxation times of low field peaks make this assessment only tentative. In NMR MTX #2, the dominant peaks in the  $\delta 4.2$  to  $\delta 3.4$  range are greatly attenuated, unquestionably due to the removal of large sugar ring type compounds in the H<sub>2</sub>O C-18 cleanup.

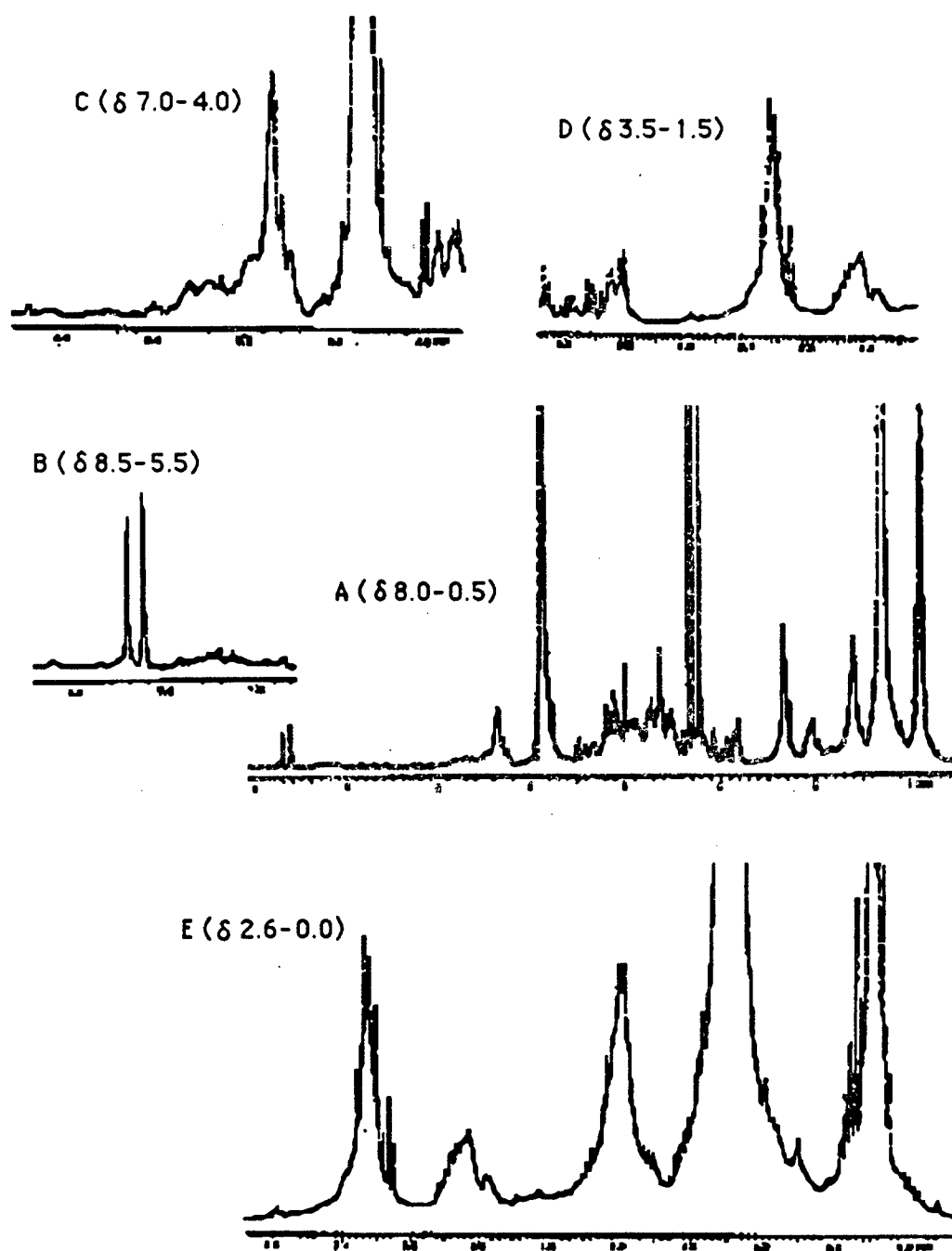


Figure 23. NMR spectra of fraction MTX #2.

Large C-18:3 lipid peaks at  $\delta$  5.35, 2.82, 2.05, 1.60, 1.30 and 0.95 visible in NMR MTX #2 may have been due to material picked up from the C-18 column as they were not present to this extent in NMR



MTX #1. Increased concentration of a prenyl-lipid type compound (indicated by ring peaks at  $\delta 7.7$  and  $\delta 7.6$ ) are seen in NMR MTX #2. We have found this particular compound repeatedly in large concentration in pigment extractions and it appears light green at low concentrations. Finding this compound present at this stage of the cleaning procedure substantiates the lipid nature of the toxin.

The NMR spectrum of NMR MTX #2 is strikingly similar to the published  $^1\text{H}$  spectrum of Yasumoto. The low field signals at  $\delta 4.5$  to  $\delta 6.0$  coincide and the  $\delta 4.5$  to  $\delta 3.0$  profile is very similar, while peaks in  $\delta 2.4$  to  $\delta 2.8$  range are completely eliminated. The small section of peak  $\delta 4.6$  to  $\delta 4.2$  are identical. The enhancement of high field peaks is undoubtedly due to lipid pickup. Further cleaning of the toxin is in process.

#### Delivery of toxins:

The following were delivered to USAMRIID prior to this report:

Lot No	Kind	Contract Item No	Amt	Date
Lot GT350	GT-1 Ext	Item 0001BM	1.0 ml	8Mar89
Lot GT350	GT-4 Ext	Item 0001BN	1.0 ml	8Mar89
Lot GT175	GT-1 Ext	Item 0001BO	0.1 ml	8Mar89
Lot GT175	GT-4 Ext	Item 0001BP	1.0 ml	8Mar89
Lot GT175	GT-4 Ext	Item 0001BQ	400 0ml	3Apr89
Lot GT175	GT-4(25%)	Item 0001BR	3.0 ml	27May89
Lot GT175	GT-4 Ext	Item 0001BS1-4	400 0ml	2Jan90

#### Travel performed during this period:

The Fourth International Conference on Toxic Dinoflagellates was attended by Donald R. Tindall, Jeffrey Bomber and Donald M. Miller. Three posters were presented and papers were accepted for publication. The titles are as follows:

NMR Spectroscopy of chlorophyll(s)-a isolated from *Gambierdiscus toxicus*. Donald M. Miller, Donald R. Tindall and Charles W. Venable

Resolution of the pigment composition and low-light response of fourteen clones of *Gambierdiscus toxicus*. Jeffrey W. Bomber, Donald R. Tindall, Charles W. Venable and Donald M. Miller.

Toxicity of *Ostreopsis lenticularis* from the British and United States Virgin Island. Donald R. Tindall and Donald M. Miller.

The principal investigator visited Queensland Institute of Technology, Brisbane Australia during the month of October and the first two weeks of November, 1989. During the visit an attempt was made to accumulate several clones of *G. toxicus*. Unfortunately the weather was not as cooperative as it could have been. However, a couple of the subcultures have survived the travel, including the polynesian strain.

In addition to didactic presentations, an ileum preparation was setup and demonstrated for the group.

#### Discussion of Results

One of the major problems that we have encountered in this experiment is that the cultures in the lowest light intensity (378 lux) did not produce cell numbers above 700 cells/ml in a reasonable time period at 28°C and 25°C (< 2 months). In addition, 378 and 678 lux cultures could not support any growth at 22°C. Therefore, in order to avoid cellular degradation by bacteria we are harvesting these cultures at lower densities. For this reason it is difficult to compare these cultures directly with the others which are harvested at  $1,100 \pm 200$  cells/ml. Consequently, we are using growth pattern as much as population density as our criterion for determining the point of harvest. Thus, all cultures have been harvested in what we determine to be mid log phase based on linear growth/time plots. By this method we insure that although cell numbers and external nutrient conditions may vary at the point of harvest, the relative kinetics of growth are the same and differ only in their magnitude.

Microalgae tend to produce more lipid and carbohydrates per cell than protein in unfavorable environments.<sup>[63,64,65]</sup> This is thought to be due to the cells preparing storage products for probable dormancy (e.g., encystment) periods.<sup>[66]</sup> The increase in carbohydrate at low light intensities may reflect this trend.

However, lipids are most concentrated at 1,999 lux. This is partially explained by the large increase in pigments at this light intensity.

The decrease in chlorophyll production under bright light is consistent with previous studies.<sup>[67]</sup> Interestingly, the growth rate data do not clearly indicate the beginning of growth saturation whereas the pigment data do. The chlorophyll to carotenoid ratio decreased at the highest light intensity at 28°C. This implies that at the high light intensity used (3350 lux), more carotenoids are being produced to protect the chlorophyll. Perhaps in the final run at 3782 and 4300 lux at 28°C this pattern will become clearer. Saturation is always suspect when more accessory pigments are made per cell relative to chlorophyll<sup>[67]</sup> as the accessory pigments (or other photosynthetic modulators) are produced to protect chlorophyll against photooxidation.

The cooler temperatures are obviously stressful for clone 175, given the decline in pigment production. The increase in toxin production with decreasing temperature (or other stress) is a pattern consistent with the production of allelopathic polyethers in bacteria<sup>[68,69]</sup> and other bioactive metabolites in dinoflagellates.<sup>[70]</sup> The fact that clone 175 is physiologically light tolerant would explain why light has relatively unexciting impacts on its toxin production. However, because it is greatly susceptible to decreasing temperature, from the evolutionary standpoint improved toxin production at this point is highly advantageous. That is, assuming that toxins produced by clone 175 affect other algae. In fact, there is good evidence to suggest that there are allelopathic roles for extracts from *G. toxicus*.<sup>[57,71]</sup> The observed pattern of toxin production would enable *G. toxicus* to maintain its ecological position in the benthos under temperature stress, despite the presence of other faster-growing, more temperature-tolerant algae. The hypothesis regarding allelopathy is worthy of further testing and is exciting basic research.

Putting allelopathic considerations aside, the reader should also keep in mind that the biochemical pathways of polyether and carotenoid production probably begin with the same carboxylic acid

precursors, e.g., acetate. Therefore, an increase in maitotoxin production during decreased carotenoid production may indicate the existence of an acetate shunt. Carotenoid and toxin contents are negatively correlated. If possible, we will explore the relationships between acetate, toxin and carotenoids in more detail.

It appears that we have in fact almost eliminated production of maitotoxin at some light intensities. We have injected mice with low light intensity samples at 128 times the usual LD<sub>50</sub> dose for this clone without adverse effects (512 µg). Perhaps there is more of the less potent, lipid-soluble toxin ("ciguatoxin") present on the ends of the bell shaped toxigenesis curve (Fig. 10). Future ileum and HPLC runs may help us determine this. In addition, some of the toxin may be leached. Bomber<sup>[57]</sup> was able to detect toxin in the medium of some cultures of *G. toxicus*. In the previous quarterly we also reported on the poor condition of the cells grown under low light. However, as all cultures are harvested in log phase, leaching is probably not that great a problem. Actively growing cells tend to conserve photosynthates for growth. In contrast, it is the stationary phase cultures which tend to leach a variety of compounds, often for ecological purposes.<sup>[72]</sup>

The next important question is, what aspect of the light intensity effect is causing the potency differences? It was surprising not to find toxin production closely associated with the production of lipids, considering that maitotoxin is supposedly a lipid. In other related work (Work in progress), toxin production over time in batch culture was not closely related with lipid production either. Therefore, toxin production is probably driven by other processes, such as temperature stress. Toxigenesis could also be linked with the population density of a culture (Fig. 11) which in turn may regulate ammonium uptake (Fig. 12). This link also supports the hypothesis regarding allelopathy. An increase in the population of any competing cells would provide both physical and chemical stimuli to *G. toxicus*. These stimuli would probably begin with ammonium deficiency because it is perhaps the most ephemeral nutrient. Improved toxin production in response to this stimulus may lead to

the death of competing algae as maitotoxin is leached from *G. toxicus*. In order for this hypothesis to be valid the stimulus-response time would have to be rapid because ammonium depletion and the termination of toxigenesis coincide.

Ammonium uptake rates and cell density are both of course light dependent. Suppose that maitotoxin facilitates the transport of cations like  $\text{NH}_4^+$  across the cell membrane. If true, then more toxin would need to be produced by a cell when the population increases in order for it to preserve its fast uptake of ammonium and remain competitive with other cells. This would then explain the strong association. Or, the process may respond to environmental cues. For example, ammonium uptake decreased in the brightest light used because there was simply little ammonium left in the medium. Interestingly, this is precisely the same point at which toxin production decreased. Certainly, the complete data set will allow more rigorous hypothesis testing to be conducted. In addition, we have initiated a new experiment examining toxin production at several nitrogen/phosphorous atomic ratios in order to possibly improve toxin production due to nutrient limitation.

Perhaps the most valuable information gathered is that we now know that maitotoxin production can be manipulated. An earlier, less detailed report stated that it could not [71]. When the present experiment is terminated we will be able to further improve toxin production by adjusting the light intensity of our mass cultures to the preferred intensity. A light intensity between 1000 and 2000 lux will foster the greatest toxin production (Fig. 10). In addition, if kept below 2000 lux the culture will still be at less than maximum lipid production (Fig. 3). Also, if harvesting is maintained in log phase, interfering lipids will be produced at their nominal levels and these features will greatly improve toxin purification. Again, we also now know that a reduced temperature appears to improve toxin production. It will be most interesting to learn if this lower temperature is stimulating an increase in maitotoxin production or perhaps an initiation or an increase in ciguatoxin synthesis. An HPLC method for detecting ciguatoxin has been recently reported by

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Yasumoto.<sup>[73]</sup> Consequently, we are reserving portions of these samples for possible future HPLC analysis.

If the data continue to support the toxigenesis/ammonium relationship then we could also improve toxin production by controlling the concentration of this nutrient.

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## GLOSSARY

ACN	Acetonitrile
ACN-insoluble	Acetonitrile insoluble fraction
ACN-soluble	Acetonitrile soluble fraction
ANOVA	Analysis of variance
CTX	Ciguatoxin
DEPT	Distortionless Enhancement Polarization Transfer Plot
DMSO	Dimethyl sulfoxide
ESAF	Ether soluble acetone filtrate
ESAP	Ether soluble acetone precipitate
GT-1	One of two toxins found in CTX group from <i>G. toxicus</i> .
GT-2	One of two toxins found in CTX group from <i>G. toxicus</i> .
GT-3	One of two toxins found in MTX group from <i>G. toxicus</i> .
GT-4	One of two toxins found in MTX group from <i>G. toxicus</i> .
HPLC	High Pressure Liquid Chromatography
IEU	Ileum Equivalent Units
MTX	Maitotoxin
MU	Mouse units
NMR	Nuclear Magnetic Resonance
P-	Abbreviation for Preparative
PPM	Parts per million
PSS	Physiological Saline Solution
SK	Acronym for Sep Pak separation of toxins
SP-	Abbreviation for Semipreparative
TEA	Tetraethylammonium
TLC	Thin layer chromatography
WSAF	Water soluble acetone filtrate
WSAP	Water soluble acetone precipitate
XTL	Acronym for crystalization step

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